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Title: Regulation of endothelial dynamics by PGC-1α relies on ROS control of VEGF-A signaling.

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Abstract: Peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) is a regulator of mitochondrial metabolism and reactive oxygen species (ROS) that is known to play a relevant role in angiogenesis. Aims: This study aims to investigate the role of ROS on the regulation by PGC-1α of angiogenesis. Methods and Results: We found that endothelial cells (ECs) from mice deleted for PGC-1α display attenuated adhesion to the extracellular matrix, together with slower and reversible spreading. Structural analysis demonstrates unstable formation of focal adhesions, defective cytoskeleton reorganization in response to cellular matrix adhesion, cell migration and cell-cell adhesion. Confluent cultures showed also a reduction of membrane bound VE-cadherin, suggesting defective inter-cellular junction formation. Functional consequences included impaired directional migration, and enhanced tip phenotype in aortic explants sprouting assays. At the molecular level, PGC-1α-deleted ECs exhibit a constitutive activation of the vascular endothelial growth factor-A (VEGF-A) signaling pathway and a defective response to VEGF-A. All these alterations are partially reversed by administration of the antioxidant EUK-189. The contribution of mitochondrial ROS and NOX activation was confirmed using a mitochondrial targeted antioxidant (MitoTEMPO) and a NOX inhibitor (VAS-2870). These results indicate that elevated production of ROS in the absence of PGC-1α is a key factor in the alteration of the VEGF-A signaling pathway and the capacity of endothelial cells to form stable interactions with other endothelial cells and with the extracellular matrix. Our findings show that PGC-1α control of ROS homeostasis plays an important role in the control of endothelial response to VEGF-A.

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Madrid, 15th of January, 2016

Dear Editor,

We respectfully submit the revised version of the manuscript, entitled *"Regulation of endothelial dynamics by PGC-1α relies on ROS control of VEGF-A signaling"* Manuscript No.: FRBM-D-15-00688 to be considered for publication in as an original article in *Free Radical Biology & Medicine.* In this revised version we hope to have fully addressed all the editor's and reviewer's criticisms to the original manuscript. We would like to thank the reviewers for their contribution to the improvement of the quality of the manuscript.

In this study we show that PGC-1α-deficient endothelial cells interact poorly with ECM and neighboring cells, and exhibit a reduced response to VEGF-A. Further analysis suggested that the observed phenotype could be due to the excessive ROS levels, previously reported to be a hallmark of PGC-1α inactivation. Indeed, antioxidant treatment reverted both the insensitivity to VEGF-A stimulation and partially corrected the phenotype, indicating that ROS-dependent alterations in VEGF-A signaling are likely to be important in the vascular instability associated with the absence of PGC-1α.

PGC-1α has been previously shown regulate angiogenesis, however vascular problems relating to PGC-1α activity were thought to reflect PGC-1α regulation of VEGF-A expression. Furthermore, it has been proposed that PGC-1α makes endothelial cells insensitive to pro-angiogenic factors and works essentially as an anti-angiogenic factor itself, in endothelial cells. Importantly, we show that PGC-1α plays a crucial role in the formation of stable vascular structures and its activity is necessary for an effective response to VEGF-A signaling. Thus, we propose that the vascular anomalies found in PGC-1α$^{-/-}$ mice relate to the association of cell quiescence with ROS control. Oxidative metabolism reduces ROS levels and favors cell quiescence and in the case of the vascular endothelium, inability to induce it in the absence of PGC-1α results in vascular instability.

We believe our results are of interest to the readership of *Free Radical biology & Medicine* since they suggest that many of the microvascular anomalies that are characteristic of metabolic dysfunctions, and in particular of diabetic retinopathy, may be related to the low levels of PGC-1α in the vascular endothelium. Further, our findings provide new mechanistic insights into vascular biology and disease, because in

patients with metabolic disease, reduced PGC-1α levels are likely to cause a ROS-dependent inactivation of key VEGF-A signaling pathway nodes, and this inactivation can be relevant in the development of microvascular complications.

Below we provide a detailed account of all the changes made to the manuscript in response to the reviewers' comments. We believe this new version answers the reviewer's criticisms, and that the scientific interest of the work has been increased. We believe that our study makes an important contribution toward understanding the connections between metabolism and oxidative stress.

Sincerely yours,

María Monsalve

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Point by point answer to reviewers:

Reviewer 2:

1.- The authors used a NOX inhibitor rather than an antioxidant to confirm their findings.

Answer. We have no used a mitochondrial-targeted antioxidant (MitoTEMPO) to evaluate the contribution of mitochondrial ROS. The results have been incorporated in the new Figure 6.

2.- This data was buried in a Supplement and not even mentioned in the Abstract.

Answer. The new data, together with the previous experiments on NOX activity are now part of Figure 6. The main conclusión has been included in the Abstract and reads: "The contribution of mitochondrial ROS and NOX activation was confirmed using a mitochondrial targeted antioxidant (MitoTEMPO) and a NOX inhibitor (VAS-2870)."

3.- The blots using the NOX inhibitor were not at all convincing for the data shown. There appeared to be artifacts on the figures as well. The quantitation on the bands is not convincing.

Answer. We have the extended text in the results section and in the Figure 6 legend aiming to clarify the results and the conclusions. The new text reads as follows:

PGC-1α has been shown to induce the expression of several mitochondrial antioxidant proteins, and its absence results in enhanced mitochondrial superoxide levels. Concomitantly, PGC-1α also regulates the expression of important extra-mitochondrial antioxidants, like catalase. In order to validate the contribution of mitochondrial ROS to the observed alteration in VEGF signaling, we treated PGC-1$\alpha^{-/-}$ MLEC with a mitochondria-targeted SOD mimetic, MitoTEMPO and evaluated the effect on VEGF-A signaling. We found a significant reduction in the basal levels of phosphorylation of VEGFR2 and AKT, as well as a reduction in the basal levels of NICD (Fig. 6A, central panel). VEGF-A treatment also resulted in an enhanced fold induction of VEGFR2 and AKT phosphorylation, as well as in the levels of NICD upon stimulation (Fig. 6A, bottom panel). These results suggest that there is a relevant contribution of mitochondrial ROS production to the observed phenotype.

Finally, since hydrogen peroxide dependent inactivation of VEGF signaling pathway has been proposed to be mediated by NOX activity, we tested whether NOX inhibition would be able to restore VEGF-A signaling in PGC-1$\alpha^{-/-}$ cells, and we found that pretreatment with the general NOX inhibitor VAS-2870 also resulted in significant reduction in the basal levels of phosphorylation of VEGFR2 and AKT and an enhanced sensitivity (fold induction) of the VEGFR2 receptor to VEGF-A dependent phosphorylation, that correlated with and enhanced AKT phosphorylation, supporting the involvement of NOX activity (Fig. 6B).

Figure 6. Both detoxification of mitochondrial ROS and NOX inhibition restores VEGF signaling in PGC-1$\alpha^{-/-}$ MLECs. PGC-1$\alpha^{-/-}$ MLECs where pre-treated with the mitochondria-targeted SOD mimetic MitoTEMPO (10 μM) for 3 h **(A,** n=5**)** or with a general NOX inhibitor (VAS-2870, 20 μM) for 2h (**B,** n=3), then VEGF-A was added and downstream signaling (VEGFR2, NICD and AKT) was analyzed by western blot (n=5). Top panels show representative western blots. Central panels show the quantitative analysis of data at time 0, prior to VEGF-A addition. The bottom panels show the fold induction upon VEGF-A addition, values are normalized for those at time 0, that have been assigned the arbitrary value of 1. Data are means ±SD (*) $p \leq 0.05$ vs. control.

4.- The bands in the new figures were not labeled.

Answer. We have now labeled the bands as suggested.

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4.- The bands in the new figures were not labeled.

Answer. We have now labeled the bands as suggested.

Highlights

. We evaluated the role of PGC-1α on endothelial cell dynamics.

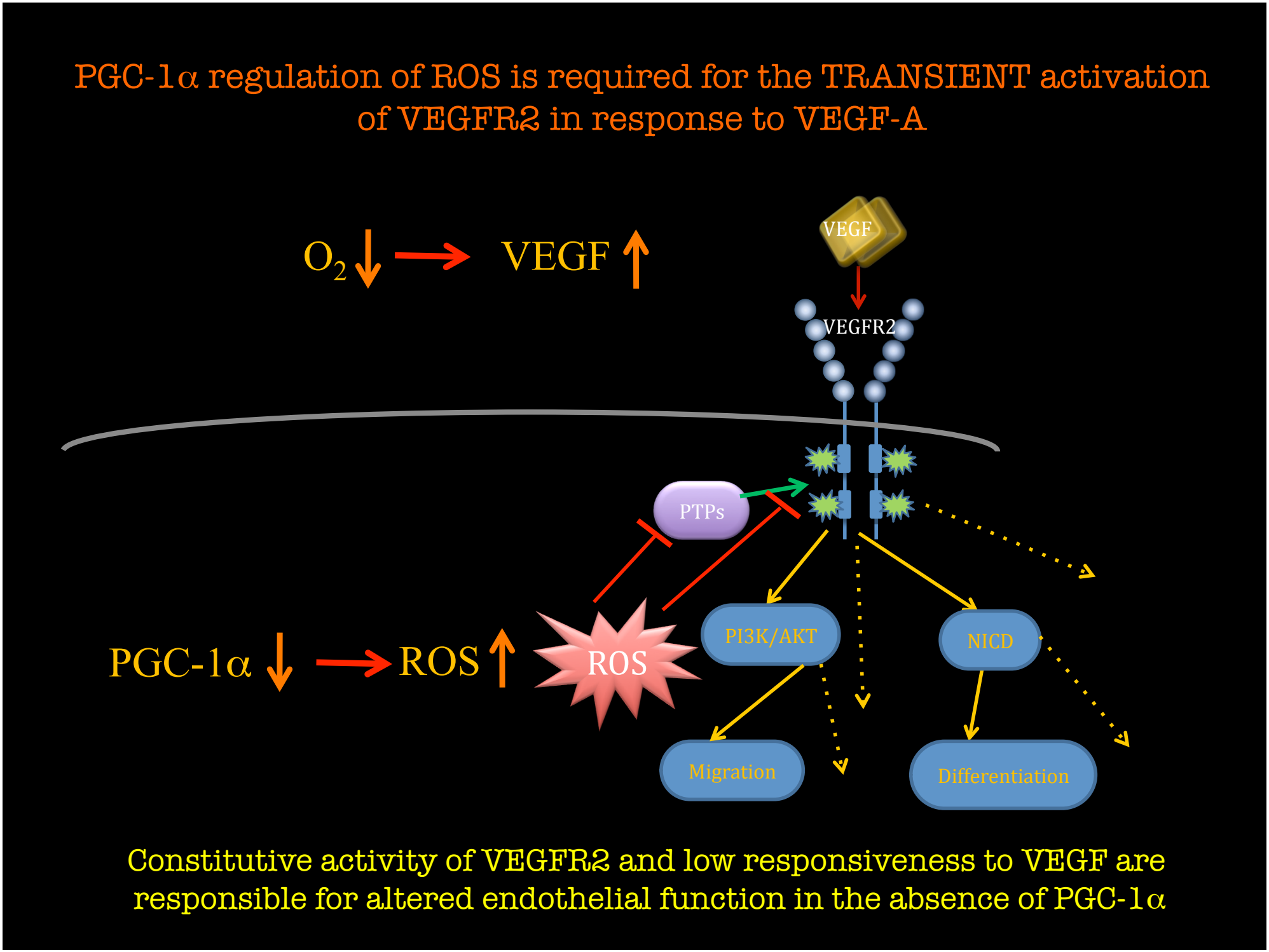
. PGC-1$\alpha^{-/-}$ endothelial cells display a constitutively activated phenotype.

. PGC-1$\alpha^{-/-}$ endothelial cells show a constitutive activation of VEGFR2.

. Antioxidant treatment of PGC-1$\alpha^{-/-}$ endothelial cells restores VEGFR2 sensitivity.

. Antioxidant treatment of PGC-1$\alpha^{-/-}$ endothelial cells restores angiotube formation.

Graphical Abstract (for review)



Regulation of endothelial dynamics by PGC-1α relies on ROS control of VEGF-A signaling.

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Short title: PGC-1α controls VEGF-A signaling.

Key words: vascular endothelium, VEGF-A, PGC-1α, oxidative stress, mitochondria.

Total Word Count: 6.073

Abstract

Peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) is a regulator of mitochondrial metabolism and reactive oxygen species (ROS) that is known to play a relevant role in angiogenesis. *Aims:* This study aims to investigate the role of ROS on the regulation by PGC-1α of angiogenesis. *Methods and Results:* We found that endothelial cells (ECs) from mice deleted for PGC-1α display attenuated adhesion to the extracellular matrix, together with slower and reversible spreading. Structural analysis demonstrates unstable formation of focal adhesions, defective cytoskeleton reorganization in response to cellular matrix adhesion, cell migration and cell-cell adhesion. Confluent cultures showed also a reduction of membrane bound VE-cadherin, suggesting defective inter-cellular junction formation. Functional consequences included impaired directional migration, and enhanced tip phenotype in aortic explants sprouting assays. At the molecular level, PGC-1α-deleted ECs exhibit a constitutive activation of the vascular endothelial growth factor-A (VEGF-A) signaling pathway and a defective response to VEGF-A. All these alterations are partially reversed by administration of the antioxidant EUK-189. The contribution of mitochondrial ROS and NOX activation was confirmed using a mitochondrial targeted antioxidant (MitoTEMPO) and a NOX inhibitor (VAS-2870). These results indicate that elevated production of ROS in the absence of PGC-1α is a key factor in the alteration of the VEGF-A signaling pathway and the capacity of endothelial cells to form stable interactions with other endothelial cells and with the extracellular matrix. Our findings show that PGC-1α control of ROS homeostasis plays an important role in the control of endothelial response to VEGF-A.

Introduction

While caloric restriction boost in the organism the use of mitochondria as a source of ATP, overfeeding tends to decrease the use of mitochondria as a source of ATP and leads to a preponderance of anaerobic glycolysis in a variety of tissues [1, 2]. This decrease in mitochondrial function is mediated by the down-regulation and functional inactivation of key metabolic regulators like the transcriptional coactivator Peroxisome proliferator-activated receptor gamma coactivator-1-alpha (PGC-1α) [3, 4], a master regulator of genes involved in oxidative metabolism. Loss of PGC-1α activity results in an increase of mitochondrial-derived reactive oxygen species (ROS) [5], and elevated ROS levels (oxidative stress) have been found in the majority of vascular complications associated with metabolic disorders.

The importance of protecting the body from the most common metabolic disorders, including obesity and type 2 diabetes cannot be overstated, and their prevalence continues to increase yearly and World-wide. Dysfunctions of the human vascular tree are the major source of morbidity and mortality. Generally, the damaging effects can be separated into macrovascular (coronary artery disease, peripheral arterial disease, and stroke) and microvascular (nephropathy, neuropathy, and retinopathy) injury [6]. Diabetic retinopathy is one of the most common microvascular complications of diabetes, and is a leading cause of ~10,000 new cases of blindness every year in the United States alone [7].

Reduced mitochondrial activity has been amply reported as a hallmark of type 2 diabetes [8, 9] and reduced activity of PGC-1α in diabetic subjects has been show in several tissues including the skeletal muscle [2]. However, elevated mRNA levels of PGC-1α have been found in the liver of type 2 diabetic subjects and it has been proposed that elevated PGC-1α in diabetic subjects increases gluconeogenesis pointing to a putative detrimental role of PGC-1α in type 2 diabetes. More recently, it has also been proposed that PGC-1α activation in the retina may be involved in type 2 diabetic retinopathy, through the induction of VEGF-A levels [10, 11].

Previous results from our group showed that PGC-1α was present in vascular endothelial cells (ECs), and its levels were reduced by hyperglycemia. Furthermore, PGC-1α could coordinate ECs oxidative metabolism and antioxidant capacity [12], suggesting that PGC-1α could play a key role in the physiology of the vascular endothelium. ROS (ie hydrogen peroxide) are acknowledged as important signaling mediators regulating cell proliferation and migration [13]. Additional studies from our laboratory showed that, at least *in vitro*, PGC-1α was a negative regulator of EC migration indicating that PGC-1α activity could be important for the control of vascular stability and angiogenesis. Accordingly, in response to angiogenesis mediators, PI3K-AKT activation resulted in the down-regulation of PGC-1α leading to increased mitochondrial ROS levels (oxygen superoxide and hydrogen peroxide) and enhanced EC migration [14]. More recently, it has been shown that PGC-1α deficient mice show defective induction of VEGF-A in response to ischemia and impaired angiogenesis [15, 16]. However, the molecular mechanisms involved, other than altered endothelial cell migration where largely unknown. Importantly, hydrogen peroxide is increasingly recognized as a regulator of VEGF-A signaling [17, 18]. Since VEGF-A activity is a crucial factor in the regulation of endothelial motility and interaction with other cells and with the basal membrane. In this study we aimed to characterize the role of PGC-1α as a regulator of ROS homeostasis in the control of endothelial adhesion dynamics and VEGF-A signaling.

We found that ECs deleted for PGC-1α had a reduced capacity to interact with each other and with the extracellular matrix, and were unable to form angiotubes *in vitro*. Furthermore, PGC-1α deleted ECs moved faster but in a non-coordinated manner, and displayed a tip-like phenotype. Importantly, their response to VEGF-A was impaired. These characteristics would be consistent with a reduced capacity to form a stable vasculature. Crucially, this phenotype could be partially rescued by antioxidant treatment, suggesting that excessive hydrogen peroxide production is likely to play an important role in vascular stability.

Material and Methods

Animal handling. C57BL6 PGC-1$\alpha^{+/+}$ and PGC-1$\alpha^{-/-}$ were used. C57BL/6 PGC-1$\alpha^{-/-}$ mice were originally provided by Dr. Bruce Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School) and following embryo transfer, a colony was established at the CNIC and IIB SPF animal facilities. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the CNIC (N^{o} PI 19/09) and the CSIC (SAF2012-37693). All procedures conformed to the Declaration of Helsinki and the NIH guidelines for animal care and use (NIH publication No. 85-23). Animals were maintained at a constant temperature of 21+/- 1°C and on a 12-hour light–dark cycle. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the CNIC and the CSIC. All procedures conformed to the Declaration of Helsinki and the NIH guidelines for animal care and use (NIH publication No. 85-23). Mice were euthanized by carbon dioxide inhalation or cervical dislocation.

Cell culture. Bovine Aortic Endothelial Cells (BAECs) and Mouse Lung Endothelial Cells (MLECs) were obtained and cultured as described [14]. Bovine Aortas were obtained from an authorized slaughterhouse (El Matadero S.C.M., Colmenar Viejo, Madrid, Spain). Adenovirus overexpression and infection protocols have been described [19].

Cell-attachment and cell spreading assays. Experiments were performed in triplicate in 96-well plates. Wells were coated with fibronectin (1 μg/ml, Sigma) or collagen I (30 μg/ml, INAMEN Biomaterials) and then blocked for 90 minutes with 1% heat-denatured BSA in PBS. MLECs were plated at 9x10^{4} cells/well for up to 1 hour, washed, fixed with 3% PFA for 1 min, permeabilized with 2% methanol for 3 min, stained with 0.5 % crystal violet in 20 % methanol for 2 min, washed with water and examined by brightfield microscopy (Nikon Eclipse 90Ti with objectives: Plan Fluor 4x/0.13 Ph1 DL, Plan Fluor 10x/0.30 Ph1 DLL, Plan Fluor ELWD 40x/0.60 Ph2 DH). Cells were classified as spread if the nucleus and cytoplasmic extensions were distinguishable.

Time lapse video imaging of scratch assays. Scratch assays were performed as previously described [14], and images were taken every 20 min for up to 30 h with a Nikon Eclipse Ti microscope (objective 4x).

Sprouting angiogenesis on aortic explants. Assays were performed as described [20].

Immunofluorescence and phalloidin staining. MLECs grown on coverslips were fixed with 3.6% PFA, permeabilized with 0.1% Triton X-100, blocked with 2% BSA and then incubated consecutively with primary antibody (α-p-paxillin or α-VE-cadherin) and a secondary antibody (αIgG rabbit ALEXA-488 conjugate), or stained with FITC-phalloidin (Molecular Probes) in PBS for 1 h. Cells were then counterstained with DAPI, mounted and examined with a confocal microscope (Nikon A1R, objective 40x) for VE-cadherin or a TIRF microscope (Leica, objective HCX Pl Apo 100x 1.46 Oil) for p-paxilin.

In vitro endothelial cell tube formation. Assays were performed essentially as described[21]. 96-well plates were coated with 40 μl of growth factor reduced (GFR) BD Matrigel per well. MLECs (17000 cells) resuspended in 100 μl of tissue culture media with 2% Horse Serum (HS) and containing, as indicated, 50 ng/ml of VEGF-A, were seeded per well. Images were taken every 20 min for up to 48 h with a Nikon EclipseTi microscope.

Vascular Endothelial Growth Factor-A (VEGF-A) treatment. MLECs were grown to confluence in DMEM-F12 medium with 20% FBS. Following overnight serum starvation o/n (0.5% FBS) the cells were first pre-incubated with 10 μM EUK-189 or 20 μM VAS-2870 for 2 h, or 10 μM MitoTEMPO for 3 h as indicated and then incubated with 12.5 ng/ml of VEGF for up to 4 h and harvested.

Protein extraction and Western blotting. Protein extraction was as previously described [19]. Protein

extracts 20-25 μg of were resolved by electrophoresis in 6-12% SDS-PAGE gels, followed by transfer to PVDF Hybond-P membranes (Amersham Biosciences) and incubated with specific antibodies against HIF-1α (#610958, BD Transduction Laboratories), VEGF-A, (VG-1, Santa Cruz), VEGF-R2 (#55B11, 1:1000), P-VEGFR2 (# 2471, 1:1000), NICD (#4147, 1:1000), AKT (#2920) and phosphorylated AKT-Ser^{473} (#4060) (1:2000), all from Cell Signaling Technology®, and β-actin (A 5441, Sigma). β-actin was used as a loading control.

Image analysis. ImageJ software was used to analyze western blots, for global determination of areas in inmunofluorescent images, and membrane/cytosol ratios of VE-cadherin. *In vitro* angiogenesis images were analyzed using the Angiogenesis Analyzer tool for Image J developed by Gilles Carpertier.

Statistics. Data are expressed as means ±SD. Statistical significance was evaluated by analysis of variance or a nonparametric test, as appropriate. Values were considered statistically significant at $p < 0.05$. $n \geq 3$ in all experiments. The number of independent experiments, for each particular experiment, is indicated in the corresponding figure legends.

Results

PGC-1α enhances endothelial adhesion to the extracellular matrix

To evaluate the role of PGC-1α during ECs interaction with the extracellular matrix (ECM), MLECs obtained from PGC-1$\alpha^{-/-}$ and PGC-1$\alpha^{+/+}$ mice were allowed to adhere to collagen I or fibronectin-coated slides for 30 min and were subsequently counted. These substrates where selected because they are the most commonly used substrates for endothelial cells grown *in vitro.* Compared with wild type MLECs, significantly fewer PGC-1$\alpha^{-/-}$ MLECs adhered to both fibronectin and collagen I (Fig.1A, top panel); although adherence of both was greater to fibronectin than to collagen matrix. To determine whether PGC-1$\alpha^{-/-}$ MLECs adhered more slowly to ECM or with less stability, a time course analysis using fibronectin was performed, and attached cells were counted. At 20 min, the number of wild-type MLECs attached reached a maximum while PGC-1$\alpha^{-/-}$ MLECs continued to adhere after 30 min (Fig. 1A, bottom panel), suggesting that matrix attachment might be less efficient in PGC-1$\alpha^{-/-}$ MLECs.

To determine whether the absence of PGC-1α reduced the initial contacts only, or also had an effect on the formation of stable interactions with the matrix, we evaluated the cell spreading process by determining the number of attached cells that had spread 1 h after plating. Results showed that whereas greater than 80 % of wild-type MLECs had spread onto the matrix, approximately 45 % of PGC-1$\alpha^{-/-}$ MLEC had done so (Fig. 1B), suggesting that PGC-1α deficiency reduced the formation of stable adhesions.

Fig. 1

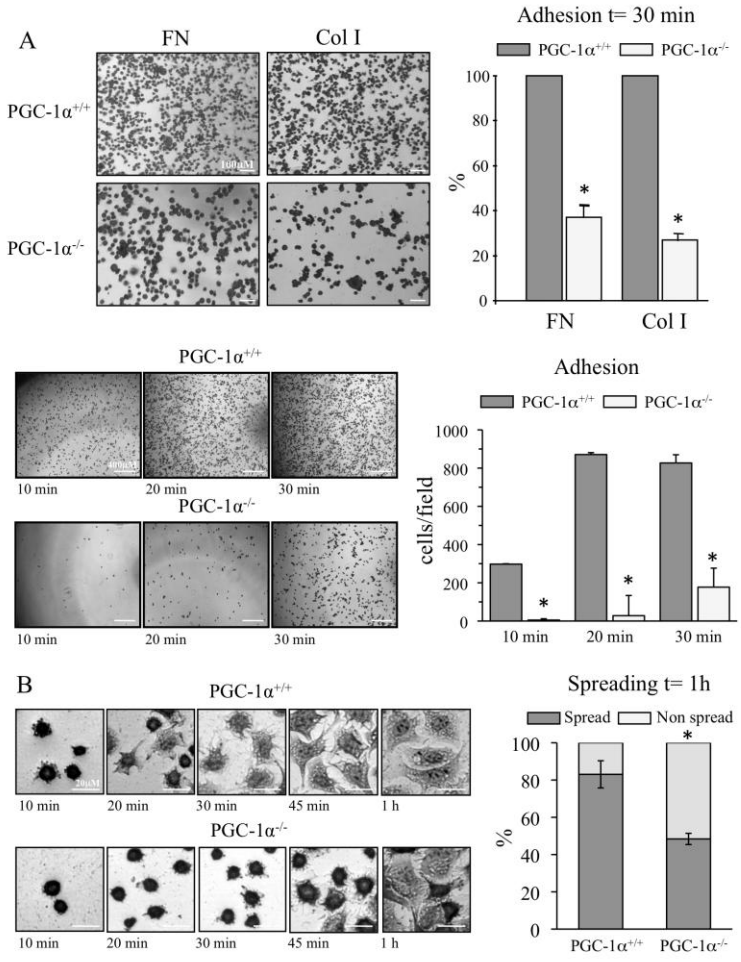

Figure 1. Endothelial cell adhesion and spreading dynamics are regulated by PGC-1α. Wild-type and PGC-1$\alpha^{-/-}$ MLECs were seeded on plates coated with fibronectin or collagen I and allowed to adhere for up to 30 min (**A**) or to spread for up to 2 h (**B**). Cells were washed fixed, bright field visualized on a microscope and counted. Left panels show images from representative experiments. Right panels show the quantifications. Data are means +SD (*) $p \leq 0.05$ vs. control. **A, top panel)** n=9. **A, bottom panel)** n=8. **B)** n=6.

Absence of PGC-1α results in unstable adhesion and spreading

To corroborate these results, we monitored the cell adhesion-spreading process using time-lapse video imaging of cell movements for 1 h after attachment to collagen I matrix. In contrast to wild-type MLECs, which displayed very limited motility after initial spreading, PGC-1$\alpha^{-/-}$ MLECs not only moved faster once attached, as has been previously described [14], but also showed incomplete and fully reversible spreading and detachment (Fig. 2A). Collectively, these results suggest that both the initial interaction with the matrix and also the formation of stable focal adhesions may be impaired in PGC-1$\alpha^{-/-}$ ECs.

To assess whether PGC-1α regulated the formation of focal adhesions, we next analyzed the focal adhesion complex-component paxillin. Paxillin is activated and phosphorylated by focal adhesion kinase (FAK) in response to integrin signaling, resulting in the formation of a focal adhesion complex. However, it must be dephosphorylated to allow the stabilization of the focal adhesion, and therefore can be used to monitor the turnover rate of newly formed focal adhesions. Analysis of phospo (p)-paxillin in MLECs that had been allowed to attach for 30 min, revealed that p-paxillin was localized to the tips of spreading filopoidia in wild-type MLECs (Fig. 2B). In contrast, PGC-1$\alpha^{-/-}$ MLECs displayed intense p-paxillin staining throughout the cell periphery, suggesting a random and highly active turnover of focal adhesions. These findings imply that the reduced adhesion capacity of PGC-1$\alpha^{-/-}$ MLECs is not due to an inability to form focal adhesion complexes but rather might be the consequence of an excessive FAK activity and high turnover rate [22]

Defective cytoskeleton dynamics in absence of PGC-1α

Cellular interactions with matrix components triggers stress fiber-formation of the polymerized actin cytoskeleton. In wild-type MLECs, F-actin was found concentrated in the extending filopodia of spreading cells 1 h after plating, and stress fibers were fully formed 2 h following plating (Fig 2B). In contrast, MLECs from PGC-1$\alpha^{-/-}$ mice exhibited dispersed F-actin foci throughout the cell periphery, and no stress fibers were detected even after 2 h (Fig 2B). To determine if this disorganization was a consequence of a deficient cell adhesion process, or could also be conspicuous in other situations, we analyzed the actin cytoskeleton of migrating and confluent cell cultures. Interestingly, while actin filaments reorganized in wild-type cells, and were about 2.5x more concentrated in the leading edge than in the retracting edge, this was barely detectable in PGC-1$\alpha^{-/-}$ MLECs (Fig. 2C). In confluent cultures, actin filaments are normally more concentrated in the cellular periphery than in the cytosol. Remarkably, this difference was more marked in PGC-1$\alpha^{-/-}$ MLEC, while mean cytosolic phalloiding stain was lower PGC-1$\alpha^{-/-}$ MLEC (Fig. 2C). These two characteristics could be related to the higher mobility of these cells. Moreover, PGC-1$\alpha^{-/-}$ MLECs were, on average, approximately 50% larger than wild-type counterparts, possibly a consequence of actin disorganization (Fig. 2D).

Endothelial cells show reduced formation of adherens junctions in absence of PGC-1α

To test whether intercellular junctions were also affected by the absence of PGC-1α, we analyzed VE-cadherin staining in confluent cultures. VE-cadherin plays a major role in the organization of intercellular adherens junctions in ECs. PGC-1$\alpha^{-/-}$ MLECs exhibited a reduction in VE-cadherin levels relative to wild-type cells; also, staining was not continuous and gaps could be detected together in zones where the membranes were not in direct contact despite the confluence of the culture (Fig. 2D, Supp. Fig. 1).

Fig. 2

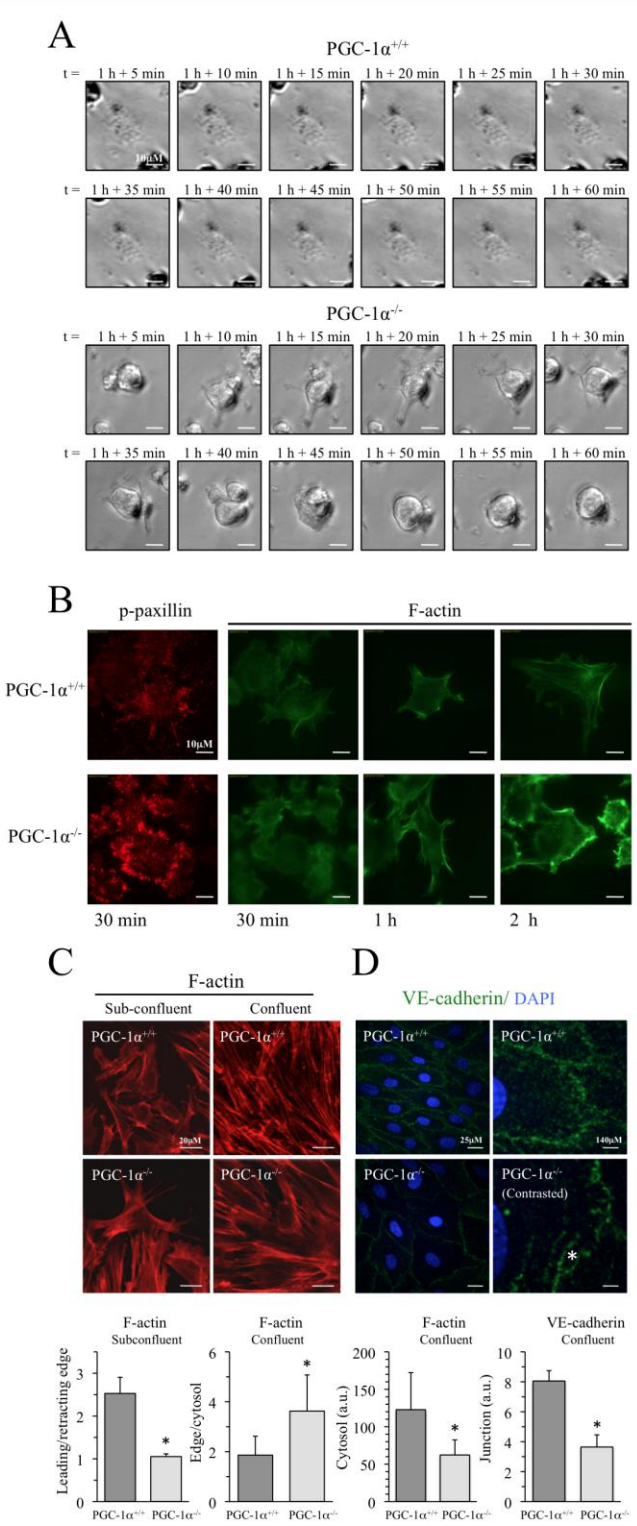

Figure 2. PGC-1α$^{-/-}$ MLEC form unstable cell-matrix and cell-cell contacts. A) Time-lapse bright field video images of live cells that have been allowed to adhere for 1 h. Images show the dynamics of representative cells at 5 min intervals for 1 h (n=3). **B)** Focal adhesion dynamics was analyzed by IF staining of cells with a p-paxillin antibody and polymerized actin filaments with phalloidin. Focal adhesions were visualized using a TIRF microscope (n=3). **C)** Wild-type and PGC-1α$^{-/-}$ MLECs actin filaments stained with phalloidin in sub-confluent and confluent MLECs and visualized with a fluorescence microscope (n=3). **D**) IF of VE-cadherin of confluent wild-type and PGC-1α$^{-/-}$ MLECs on a fibronectin-coated plate. Nuclei were stained with DAPI (n=3). Data are means +SD (*) $p \leq 0.05$ vs. control.

PGC-1α regulates endothelial cell coordination during migration

To assess how these deficiencies could impact on cell migration processes, we monitored cell motility using *in vitro* scratch assays and time-lapse video imaging. Whereas wild-type cells appeared to stream together as they migrated, we noted that the absence of PGC-1α resulted not only in an accelerated cell movement as already described [14], but also on a pronounced tendency to disperse over a larger area (Fig. 3A, lower panel; Supp. Info. Video Files). A similar effect was observed in BAECs upon PGC-1α silencing (Fig. 3A, lower panel; Supp. Info. Video Files). The Figure 3A shows only one side of the scrach in order to increase the magnification of the image and therefore be able to visualize how the cells spread out into the gap. In the images can be appreciated that cell density is lower when PGC-1α is absent or reduced while it is higher in over-expressing cells. This is not due to differences in confluency, all cultures were 100% confluent, but is mainly attributable to the bigger size (about 50%) of PGC-1α deficient cells (Fig. 2D). Nevertheless, PGC-1$\alpha^{-/-}$ fully-confuent cultures always show small gaps between cells (Fig. 2D), these could be attribute to problems in the formation of cell-cell contacts.

Fig. 3

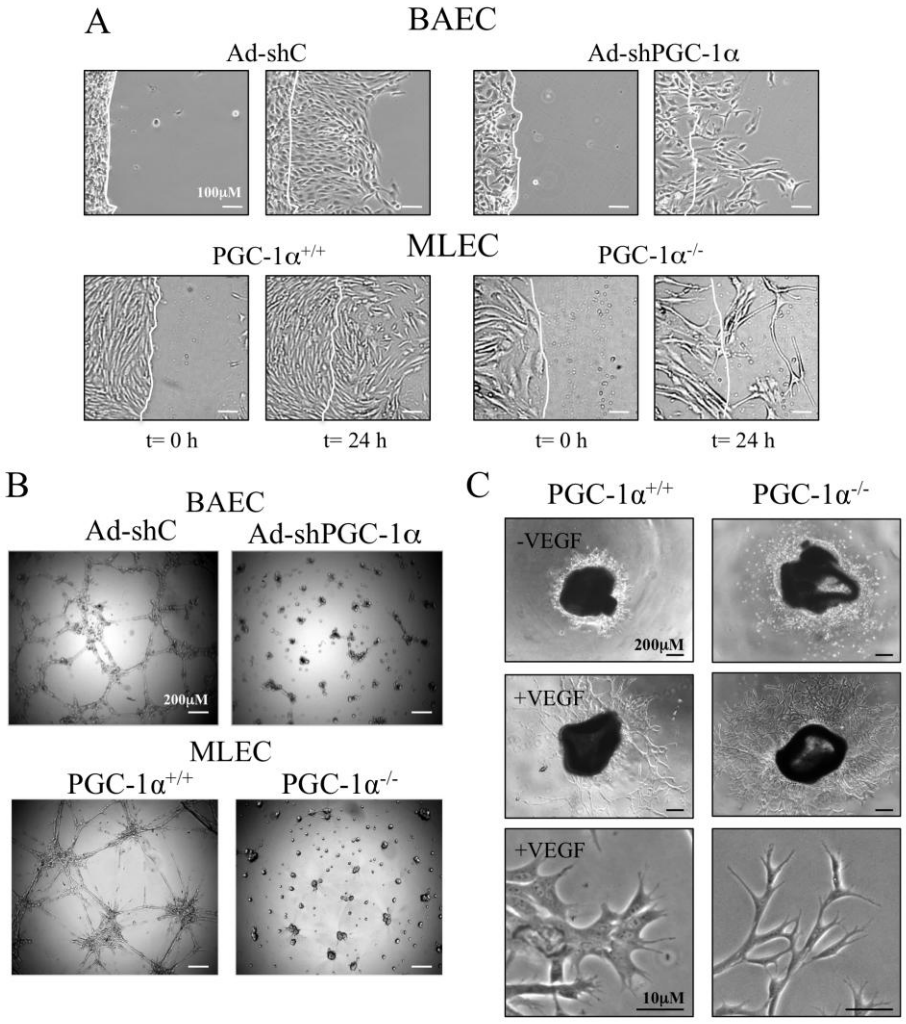

Figure 3. Lack of PGC-1α results in reduced intercellular junction/coordination during migration, and failure to form angiotubes *in vitro*. A) Scratch assays of BAECs infected with the indicated adenovirus to knock-down PGC-1α, and wild-type or PGC-1$\alpha^{-/-}$ MLECs. The migratory front is highlighted with a white line (n=6). **B)** *In vitro* angiotube assay on Matrigel using BAECs silenced for PGC-1α (upper panel), and wild-type and PGC-1$\alpha^{-/-}$ MLECs (lower panel) (n=5). **C)** Endothelial cells sprouting from aorta explants from wild-tpe and PGC-1$\alpha^{-/-}$ mice. Bright field images were taken after 5 days, the lower panels show higher magnification images of tip cells from the migratory edge (n=6). Data are means +SD (*) $p \leq 0.05$ vs. control.

Absence of PGC-1α results in impaired angiotube formation

The ability to form stable cell-to-cell contacts is not only important to ensure organized cell migration, it is also critical for the formation and stabilization of the newly formed vessel. Thus, we next tested vessel formation using an *in vitro* angiotube formation assay. Interestingly, results from silencing of PGC-1α expression in BAECs (Fig. 3B upper panel) and the absence of PGC-1α in MLECs (Fig. 3B lower panel), showed that both approaches led to an almost complete inability of ECs to form angiotubes *in vitro*, even though cells migrated and aggregated. This result suggested that deficient interactions of intercellular junctions provoked by the absence of PGC-1α are likely to be crucial for *in vivo* formation of blood vessels.

PGC-1α$^{-/-}$ endothelial cells display an enhanced tip phenotype

In a complementary approach to test EC migration and intercellular junction interactions in combination, we utilized an *ex vivo* angiogenesis assay using Matrigel-embedded aortic ring explants. We found that even in the absence of VEGF-A, PGC-1α$^{-/-}$ ECs had the capacity to migrate, while wild-type ECs could not migrate unless VEGF-A was added to the medium, and then did so in an individual manner, in the absence of interacting cells (Fig. 3C, upper panel). Moreover, we observed that, in the presence of VEGF-A, the cellular "chords" of sprouting cells exiting the aortic rings from PGC-1α$^{-/-}$ mice appeared thinner than those sprouting from wild-type aortic rings. Upon closer inspection of migratory edges, we observed that while wild-type ECs where closely packed, in several cell-wide chords, with filopodia only in the outermost migratory edge, PGC-1α$^{-/-}$ cells formed single-cell chords, and were highly elongated with extensive filopodia (Fig. 3C, lower panel). These observations suggested that in the absence of PGC-1α endothelial cells can migrate even in the absence of VEGF and they do not respond normally to VEGF-A, possibly inducing an enhanced tip-like phenotype.

PGC-1α$^{-/-}$ endothelial cells respond to antioxidant treatment enhancing VEGF-A signaling

Our previous *in vitro* results indicated that PGC-1α$^{-/-}$ MLECs exhibited a greater migration capacity compared with wild-type cells due to an overproduction of hydrogen peroxide [14]. Indeed, hydrogen peroxide is acknowledged as an important player in angiogenesis [18] and mediate EC migration and proliferation [23]. Thus, we evaluated whether the angiogenesis defects found in PGC-1α$^{-/-}$ mice could be attributed to an excess production of hydrogen peroxide. To this end, we treated PGC-1α$^{-/-}$ MLECs with an antioxidant and measured their ability to form angiotubes *in vitro*. Pre-incubation of PGC-1α$^{-/-}$ MLECs with EUK-189, a synthetic superoxide dismutase/catalase mimetic, enhanced the formation of angiotubes (Fig. 4, Supp. Fig. 2), pointing to a participation of hydrogen peroxide in capillary formation. To elucidate the molecular mechanisms that could link elevated hydrogen peroxide levels with the vascular instability observed in PGC-1α$^{-/-}$ mice, we next analyzed the response of wild-type and PGC-1α$^{-/-}$ MLECs to VEGF-A. This signaling pathway has been suggested previously to be highly sensitive to hydrogen peroxide [17, 18]. Consistently, PGC-1α$^{-/-}$ MLEC showed increased levels of phosphorylated VEGFR2, phosphorylated AKT and greater levels of NICD, compared with wild-type cells in the absence of VEGF-A (Fig. 5). Furthermore, relative to wild-type cells, the response to VEGF-A treatment was very poor in PGC-1α$^{-/-}$ MLECs in terms of VEGFR-2 induced phosphorylation, supporting the notion that PGC-1α-deleted ECs are largely insensitive to VEGF-A activation. Importantly, when PGC-1α$^{-/-}$ MLECs were pre-incubated with EUK-189, the response to VEGF-A was partially recovered, while the basal levels of phosphorylated VEGFR2, phosphorylated AKT and NICD, were reduced (Fig. 5). This observation is consistent with previous reports demonstrating the sensitivity of VEGFR-2 and its phosphatases to hydrogen peroxide [17, 18]. Moreover, these findings indicate that the response of VEGFR2 to VEGF-A requires the tight control of intracellular hydrogen peroxide levels. This control may be lost when important metabolic regulators such as PGC-1α are largely inactive [3]. mRNA levels where not significantly affected by EU-189 (Suppl. Fig. 3).

Fig. 4

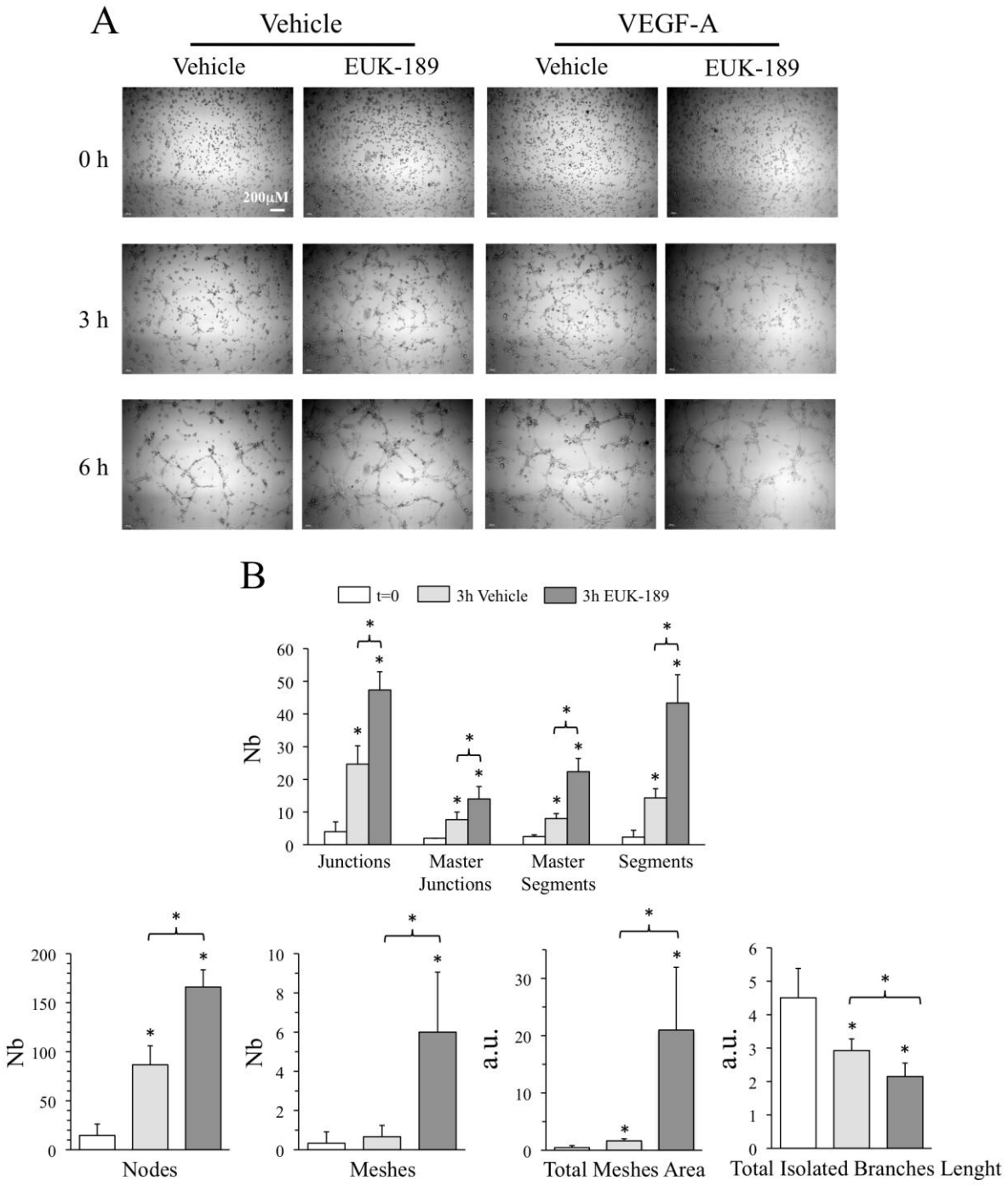

Figure 4. Antioxidant treatment improves *in vitro* angiotube formation in PGC-1α$^{-/-}$ MLECs. A) *In vitro* angiotube assay on Matrigel using PGC-1α$^{-/-}$ MLECs, treated with VEGF and/or EUK-189 (n=3). **B)** Analysis of structures formed in the angitube assay. Data are means +SD (*) $p \leq 0.05$ vs. control.

Fig. 5

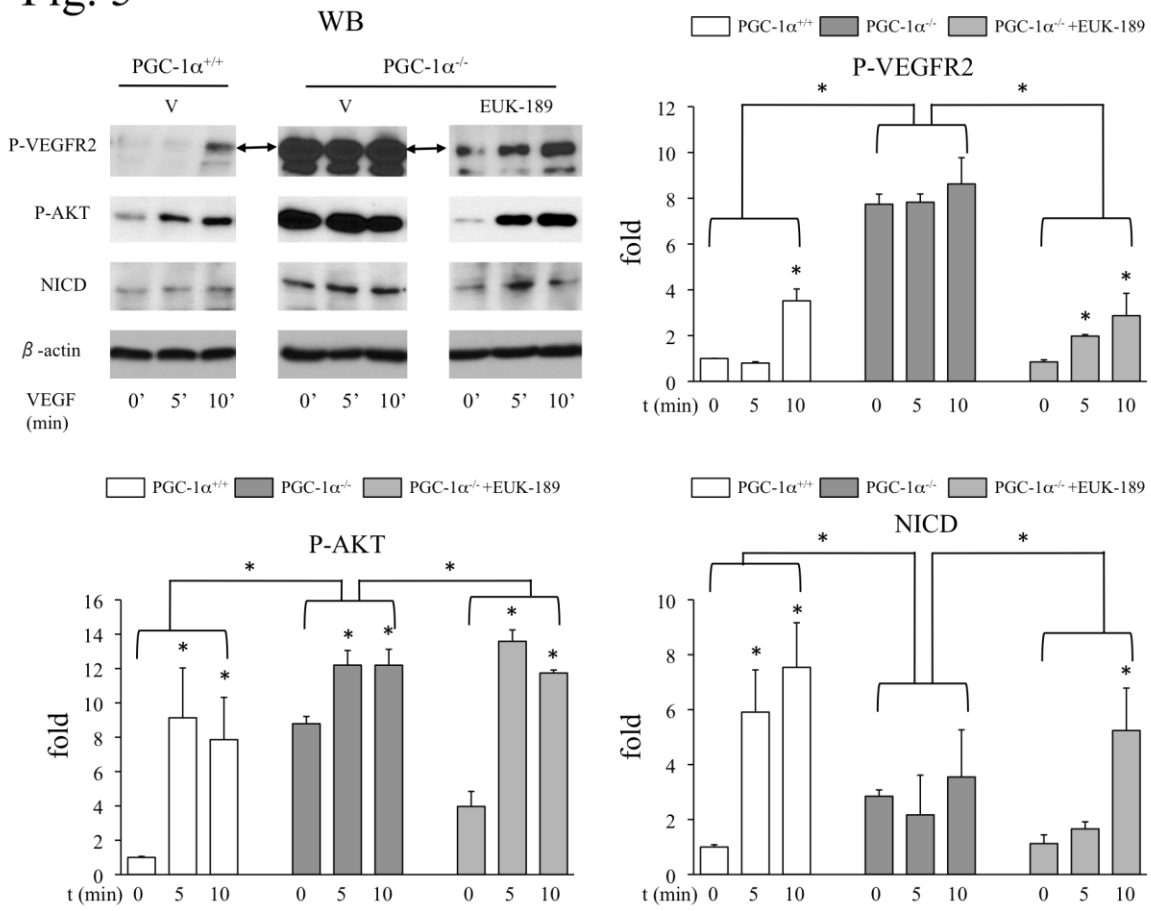

Figure 5. Antioxidant treatment restores VEGF signaling in PGC-1α$^{-/-}$ MLECs. PGC-1α$^{-/-}$ MLECs where treated with VEGF and/or EUK-189 for the indicated time periods. Western blot analysis of whole cell extracts of relevant proteins in VEGF signaling: VEGFR2, NICD and AKT (n=3). Data are means ±SD (*) $p \leq 0.05$ vs. control.

PGC-1α has been shown to induce the expression of several mitochondrial antioxidant proteins, and its absence results in enhanced mitochondrial superoxide levels. Concomitantly, PGC-1α also regulates the expression of important extra-mitochondrial antioxidants, like catalase. In order to validate the contribution of mitochondrial ROS to the observed alteration in VEGF signaling, we treated PGC-1α$^{-/-}$ MLEC with a mitochondria-targeted SOD mimetic, MitoTEMPO and evaluated the effect on VEGF-A signaling. We found a significant reduction in the basal levels of phosphorylation of VEGFR2 and AKT, as well as a reduction in the basal levels of NICD (Fig. 6A, central panel). VEGF-A treatment also resulted in an enhanced fold induction of VEGFR2 and AKT phosphorylation, as well as in the levels of NICD upon stimulation (Fig. 6A, bottom panel). These results suggest that there is a relevant contribution of mitochondrial ROS production to the observed phenotype.

Finally, since hydrogen peroxide dependent inactivation of VEGF signaling pathway has been proposed to be mediated by NOX activity, we tested whether NOX inhibition would be able to restore VEGF-A signaling in PGC-1α$^{-/-}$ cells, and we found that pretreatment with the general NOX inhibitor VAS-2870 also resulted in significant reduction in the basal levels of phosphorylation of VEGFR2 and AKT and an enhanced sensitivity (fold induction) of the VEGFR2 receptor to VEGF-A dependent phosphorylation, that correlated with and enhanced AKT phosphorylation, supporting the involvement of NOX activity (Fig. 6B).

Fig. 6

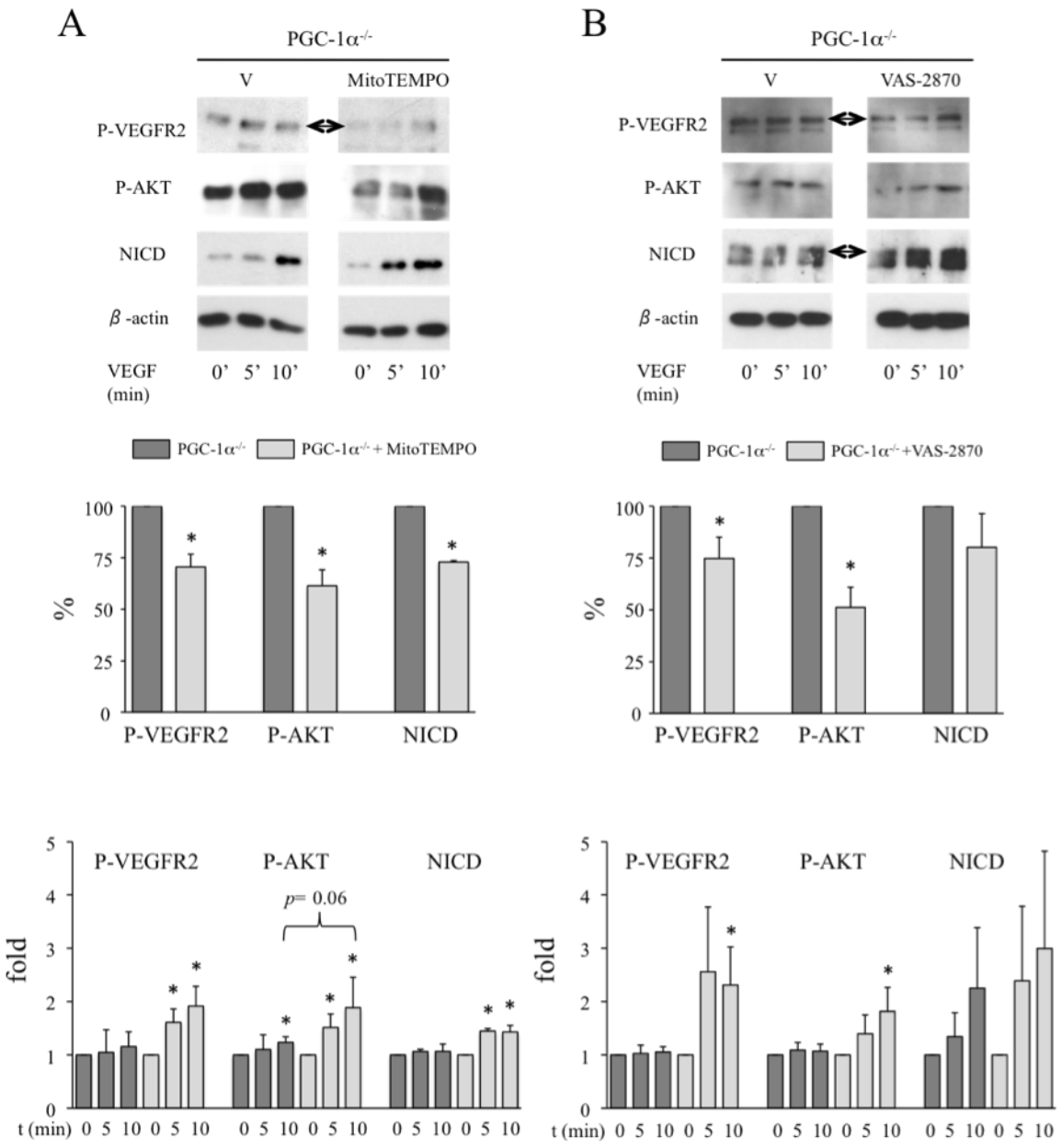

Figure 6. Both detoxification of mitochondrial ROS and NOX inhibition restores VEGF signaling in PGC-1α$^{-/-}$ MLECs. PGC-1α$^{-/-}$ MLECs where pre-treated with the mitochondria-targeted SOD mimetic MitoTEMPO (10 μM) for 3 h **(A,** n=5**)** or with a general NOX inhibitor (VAS-2870, 20 μM) for 2h (**B,** n=3), then VEGF-A was added and downstream signaling (VEGFR2, NICD and AKT) was analyzed by western blot (n=5). Top panels show representative western blots. Central panels show the quantitative analysis of data at time 0, prior to VEGF-A addition. The bottom panels show the fold induction upon VEGF-A addition, values are normalized for those at time 0, that have been assigned the arbitrary value of 1. Data are means ±SD (*) $p \leq 0.05$ vs. control.

Discussion

The results of this study show that regulation of ROS homeostasis by PGC-1α plays an important role in the control of endothelial function, in particular in the regulation by VEGF-A of the stability of the cellular and cellular-matrix contacts. We demonstrate that, in the absence of PGC-1α, the vascular endothelial cells appears to be constitutively activated, characterized by faster migrating ECs which adhere slowly to the substrate. Also, ECs spread slowly and can reverse this process and detach again. These alterations are likely due to the low stability and high turnover of the focal adhesions, an inability to form a structured f-actin cytoskeleton, and a reduced formation of VE-cadherin adherens junctions. Succinctly, ECs interact poorly with the substrate and with other cells. Clearly, these alterations are likely to be relevant in metabolic disorders where PGC-1α activity is generally found to be low, and in particular might be related to the development of diabetic retinopathy.

Reduced intercellular contacts in PGC-1α-deficient ECs resulted in the loss of coordinated cell movement in scratch assays, and a failure to form angiotubes *in vitro*. Analysis of the endothelial sprouting process from aortic rings further suggested that PGC-1α-deficient cells actively migrate even in the absence of VEGF-A, and form tip cells independently of their location, indicating a defective response to VEGF-A. Treatment of MLECs with VEGF-A *in vitro,* together with analysis of the VEGF pathway, confirmed that the absence of PGC-1α resulted in the constitutive activation of the VEGF-A signaling pathway, plus a poor response to exogenous VEGF-A. This defective response to VEGF-A may explain the constitutively-activated phenotype of PGC-1α deficient ECs.

PGC-1α deficient ECs have elevated levels of oxygen superoxide and hydrogen peroxide [5]. Given that the VEGF-A signaling pathway has been shown to be sensitive to hydrogen peroxide, and that oxidative modifications of VEGFR2 [18, 24] and its main phosphatases have been described [17], we hypothesized that elevated ROS levels found in PGC-1α-deficient ECs would be responsible for the poor VEGF-A response. Consistent with this idea, we found that antioxidant treatment largely restored normal VEGF-A response in PGC-1α deficient ECs. Furthermore, EUK-189 treatment also restored the capacity of those cells to form angiotubes *in vitro*, indicating a normalization of the inter-cellular interactions.

Long regarded as by-products of oxidative metabolism, ROS are now recognized as signaling mediators in their own right. The prototypic redox-regulated targets are the protein tyrosine phosphatases that are inactivated by hydrogen peroxide; however, identification of redox-sensitive kinases such as Src has led to the slow emergence of a role for redox regulation of tyrosine kinases by concurrent oxidative activation of tyrosine kinases. Thus, ROS fine-tune the duration and amplification of the phosphorylation signal. Importantly, the activity of VEGFR2 and two of its phosphatases, DEP1 and PTP1B, have been shown to be regulated by hydrogen peroxide [17] [24] [18]. Demonstration of the physiological relevance of these regulations has been hampered by the lack of relevant disease models, since the majority of experimental evidence derives from *in vitro* assays with artificially generated or exogenously added ROS; this is the case for VEGFR-2 signaling pathways.

PGC-1α is known to regulate the levels of mitochondrial ROS [5]. However, most recent studies stress the role played by NADPH oxidases (NOXs) in the ROS-dependent control of growth factor signaling processes [25] and less attention has been paid to the role of mitochondrial ROS. Nevertheless, although a largely neglected area of research, a body of evidence shows that elevated mitochondrial production of ROS is commonly found in cells with elevated NOX activity, and it has been shown that mitochondria can regulate NOX function and *viceversa* [26]. Additionally, most, if not all, physiological settings where ROS appear to play an important role, including angiogenesis, describe both enhanced NOX and elevated mitochondrial ROS levels [27, 28]. Furthermore, mitochondria-targeted antioxidants have been proposed to have potential therapeutic value in retinopathy [29]. Nonetheless, it remains to be determined whether PGC-1α could also directly regulate the expression or activity of NOX or alternatively of PrxII, the peroxiredoxin antioxidant that appears to modulate

VEGFR2 oxidation [18].

In conclusion, our study strongly supports the idea that PGC-1α control of hydrogen peroxide levels in endothelial cells plays a major role in the formation of stable interactions of endothelial cells with other cells and with the matrix, a process likely to be relevant in metabolic disorders where microvascular complications are prevalent, like diabetic retinopathy. Since antioxidant therapies have shown significant caveats, alternative therapies that aim to re-wire normal ROS homeostasis, boosting PGC-1α activity might represent a therapeutic approach for retinopathy patients.

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Conflict of Interest

None declared.

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Regulation of endothelial dynamics by PGC-1α relies on ROS control of VEGF-A signaling.

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Short title: PGC-1α controls VEGF-A signaling.

Key words: vascular endothelium, VEGF-A, PGC-1α, oxidative stress, mitochondria.

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Abstract

Peroxisome proliferator activated receptor γ co-activator 1α (PGC-1α) is a regulator of mitochondrial metabolism and reactive oxygen species (ROS) that is known to play a relevant role in angiogenesis. *Aims:* This study aims to investigate the role of ROS on the regulation by PGC-1α of angiogenesis. *Methods and Results:* We found that endothelial cells (ECs) from mice deleted for PGC-1α display attenuated adhesion to the extracellular matrix, together with slower and reversible spreading. Structural analysis demonstrates unstable formation of focal adhesions, defective cytoskeleton reorganization in response to cellular matrix adhesion, cell migration and cell-cell adhesion. Confluent cultures showed also a reduction of membrane bound VE-cadherin, suggesting defective inter-cellular junction formation. Functional consequences included impaired directional migration, and enhanced tip phenotype in aortic explants sprouting assays. At the molecular level, PGC-1α-deleted ECs exhibit a constitutive activation of the vascular endothelial growth factor-A (VEGF-A) signaling pathway and a defective response to VEGF-A. All these alterations are partially reversed by administration of the antioxidant EUK-189. The contribution of mitochondrial ROS and NOX activation was confirmed using a mitochondrial targeted antioxidant (MitoTEMPO) and a NOX inhibitor (VAS-2870). These results indicate that elevated production of ROS in the absence of PGC-1α is a key factor in the alteration of the VEGF-A signaling pathway and the capacity of endothelial cells to form stable interactions with other endothelial cells and with the extracellular matrix. Our findings show that PGC-1α control of ROS homeostasis plays an important role in the control of endothelial response to VEGF-A.

Introduction

While caloric restriction boost in the organism the use of mitochondria as a source of ATP, overfeeding tends to decrease the use of mitochondria as a source of ATP and leads to a preponderance of anaerobic glycolysis in a variety of tissues [1, 2]. This decrease in mitochondrial function is mediated by the down-regulation and functional inactivation of key metabolic regulators like the transcriptional coactivator Peroxisome proliferator-activated receptor gamma coactivator-1-alpha (PGC-1α) [3, 4], a master regulator of genes involved in oxidative metabolism. Loss of PGC-1α activity results in an increase of mitochondrial-derived reactive oxygen species (ROS) [5], and elevated ROS levels (oxidative stress) have been found in the majority of vascular complications associated with metabolic disorders.

The importance of protecting the body from the most common metabolic disorders, including obesity and type 2 diabetes cannot be overstated, and their prevalence continues to increase yearly and World-wide. Dysfunctions of the human vascular tree are the major source of morbidity and mortality. Generally, the damaging effects can be separated into macrovascular (coronary artery disease, peripheral arterial disease, and stroke) and microvascular (nephropathy, neuropathy, and retinopathy) injury [6]. Diabetic retinopathy is one of the most common microvascular complications of diabetes, and is a leading cause of ~10,000 new cases of blindness every year in the United States alone [7].

Reduced mitochondrial activity has been amply reported as a hallmark of type 2 diabetes [8, 9] and reduced activity of PGC-1α in diabetic subjects has been show in several tissues including the skeletal muscle [2]. However, elevated mRNA levels of PGC-1α have been found in the liver of type 2 diabetic subjects and it has been proposed that elevated PGC-1α in diabetic subjects increases gluconeogenesis pointing to a putative detrimental role of PGC-1α in type 2 diabetes. More recently, it has also been proposed that PGC-1α activation in the retina may be involved in type 2 diabetic retinopathy, through the induction of VEGF-A levels [10, 11].

Previous results from our group showed that PGC-1α was present in vascular endothelial cells (ECs), and its levels were reduced by hyperglycemia. Furthermore, PGC-1α could coordinate ECs oxidative metabolism and antioxidant capacity [12], suggesting that PGC-1α could play a key role in the physiology of the vascular endothelium. ROS (ie hydrogen peroxide) are acknowledged as important signaling mediators regulating cell proliferation and migration [13]. Additional studies from our laboratory showed that, at least *in vitro*, PGC-1α was a negative regulator of EC migration indicating that PGC-1α activity could be important for the control of vascular stability and angiogenesis. Accordingly, in response to angiogenesis mediators, PI3K-AKT activation resulted in the down-regulation of PGC-1α leading to increased mitochondrial ROS levels (oxygen superoxide and hydrogen peroxide) and enhanced EC migration [14]. More recently, it has been shown that PGC-1α deficient mice show defective induction of VEGF-A in response to ischemia and impaired angiogenesis [15, 16]. However, the molecular mechanisms involved, other than altered endothelial cell migration where largely unknown. Importantly, hydrogen peroxide is increasingly recognized as a regulator of VEGF-A signaling [17, 18]. Since VEGF-A activity is a crucial factor in the regulation of endothelial motility and interaction with other cells and with the basal membrane. In this study we aimed to characterize the role of PGC-1α as a regulator of ROS homeostasis in the control of endothelial adhesion dynamics and VEGF-A signaling.

We found that ECs deleted for PGC-1α had a reduced capacity to interact with each other and with the extracellular matrix, and were unable to form angiotubes *in vitro*. Furthermore, PGC-1α deleted ECs moved faster but in a non-coordinated manner, and displayed a tip-like phenotype. Importantly, their response to VEGF-A was impaired. These characteristics would be consistent with a reduced capacity to form a stable vasculature. Crucially, this phenotype could be partially rescued by antioxidant treatment, suggesting that excessive hydrogen peroxide production is likely to play an important role in vascular stability.

Material and Methods

Animal handling. C57BL6 PGC-1$\alpha^{+/+}$ and PGC-1$\alpha^{-/-}$ were used. C57BL/6 PGC-1$\alpha^{-/-}$ mice were originally provided by Dr. Bruce Spiegelman (Dana-Farber Cancer Institute, Harvard Medical School) and following embryo transfer, a colony was established at the CNIC and IIB SPF animal facilities. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the CNIC (N^{o} PI 19/09) and the CSIC (SAF2012-37693). All procedures conformed to the Declaration of Helsinki and the NIH guidelines for animal care and use (NIH publication No. 85-23). Animals were maintained at a constant temperature of 21+/- 1°C and on a 12-hour light–dark cycle. Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the CNIC and the CSIC. All procedures conformed to the Declaration of Helsinki and the NIH guidelines for animal care and use (NIH publication No. 85-23). Mice were euthanized by carbon dioxide inhalation or cervical dislocation.

Cell culture. Bovine Aortic Endothelial Cells (BAECs) and Mouse Lung Endothelial Cells (MLECs) were obtained and cultured as described [14]. Bovine Aortas were obtained from an authorized slaughterhouse (El Matadero S.C.M., Colmenar Viejo, Madrid, Spain). Adenovirus overexpression and infection protocols have been described [19].

Cell-attachment and cell spreading assays. Experiments were performed in triplicate in 96-well plates. Wells were coated with fibronectin (1 μg/ml, Sigma) or collagen I (30 μg/ml, INAMEN Biomaterials) and then blocked for 90 minutes with 1% heat-denatured BSA in PBS. MLECs were plated at 9x10^{4} cells/well for up to 1 hour, washed, fixed with 3% PFA for 1 min, permeabilized with 2% methanol for 3 min, stained with 0.5 % crystal violet in 20 % methanol for 2 min, washed with water and examined by brightfield microscopy (Nikon Eclipse 90Ti with objectives: Plan Fluor 4x/0.13 Ph1 DL, Plan Fluor 10x/0.30 Ph1 DLL, Plan Fluor ELWD 40x/0.60 Ph2 DH). Cells were classified as spread if the nucleus and cytoplasmic extensions were distinguishable.

Time lapse video imaging of scratch assays. Scratch assays were performed as previously described [14], and images were taken every 20 min for up to 30 h with a Nikon Eclipse Ti microscope (objective 4x).

Sprouting angiogenesis on aortic explants. Assays were performed as described [20].

Immunofluorescence and phalloidin staining. MLECs grown on coverslips were fixed with 3.6% PFA, permeabilized with 0.1% Triton X-100, blocked with 2% BSA and then incubated consecutively with primary antibody (α-p-paxillin or α-VE-cadherin) and a secondary antibody (αIgG rabbit ALEXA-488 conjugate), or stained with FITC-phalloidin (Molecular Probes) in PBS for 1 h. Cells were then counterstained with DAPI, mounted and examined with a confocal microscope (Nikon A1R, objective 40x) for VE-cadherin or a TIRF microscope (Leica, objective HCX Pl Apo 100x 1.46 Oil) for p-paxilin.

In vitro endothelial cell tube formation. Assays were performed essentially as described[21]. 96-well plates were coated with 40 μl of growth factor reduced (GFR) BD Matrigel per well. MLECs (17000 cells) resuspended in 100 μl of tissue culture media with 2% Horse Serum (HS) and containing, as indicated, 50 ng/ml of VEGF-A, were seeded per well. Images were taken every 20 min for up to 48 h with a Nikon EclipseTi microscope.

Vascular Endothelial Growth Factor-A (VEGF-A) treatment. MLECs were grown to confluence in DMEM-F12 medium with 20% FBS. Following overnight serum starvation o/n (0.5% FBS) the cells were first pre-incubated with 10 μM EUK-189 or 20 μM VAS-2870 for 2 h, or 10 μM MitoTEMPO for 3 h as indicated and then incubated with 12.5 ng/ml of VEGF for up to 4 h and harvested.

Protein extraction and Western blotting. Protein extraction was as previously described [19]. Protein

extracts 20-25 μg of were resolved by electrophoresis in 6-12% SDS-PAGE gels, followed by transfer to PVDF Hybond-P membranes (Amersham Biosciences) and incubated with specific antibodies against HIF-1α (#610958, BD Transduction Laboratories), VEGF-A, (VG-1, Santa Cruz), VEGF-R2 (#55B11, 1:1000), P-VEGFR2 (# 2471, 1:1000), NICD (#4147, 1:1000), AKT (#2920) and phosphorylated AKT-Ser^{473} (#4060) (1:2000), all from Cell Signaling Technology®, and β-actin (A 5441, Sigma). β-actin was used as a loading control.

Image analysis. ImageJ software was used to analyze western blots, for global determination of areas in inmunofluorescent images, and membrane/cytosol ratios of VE-cadherin. *In vitro* angiogenesis images were analyzed using the Angiogenesis Analyzer tool for Image J developed by Gilles Carpertier.

Statistics. Data are expressed as means ±SD. Statistical significance was evaluated by analysis of variance or a nonparametric test, as appropriate. Values were considered statistically significant at $p < 0.05$. $n \geq 3$ in all experiments. The number of independent experiments, for each particular experiment, is indicated in the corresponding figure legends.

Results

PGC-1α enhances endothelial adhesion to the extracellular matrix

To evaluate the role of PGC-1α during ECs interaction with the extracellular matrix (ECM), MLECs obtained from PGC-1$\alpha^{-/-}$ and PGC-1$\alpha^{+/+}$ mice were allowed to adhere to collagen I or fibronectin-coated slides for 30 min and were subsequently counted. These substrates where selected because they are the most commonly used substrates for endothelial cells grown *in vitro.* Compared with wild type MLECs, significantly fewer PGC-1$\alpha^{-/-}$ MLECs adhered to both fibronectin and collagen I (Fig.1A, top panel); although adherence of both was greater to fibronectin than to collagen matrix. To determine whether PGC-1$\alpha^{-/-}$ MLECs adhered more slowly to ECM or with less stability, a time course analysis using fibronectin was performed, and attached cells were counted. At 20 min, the number of wild-type MLECs attached reached a maximum while PGC-1$\alpha^{-/-}$ MLECs continued to adhere after 30 min (Fig. 1A, bottom panel), suggesting that matrix attachment might be less efficient in PGC-1$\alpha^{-/-}$ MLECs.

To determine whether the absence of PGC-1α reduced the initial contacts only, or also had an effect on the formation of stable interactions with the matrix, we evaluated the cell spreading process by determining the number of attached cells that had spread 1 h after plating. Results showed that whereas greater than 80 % of wild-type MLECs had spread onto the matrix, approximately 45 % of PGC-1$\alpha^{-/-}$ MLEC had done so (Fig. 1B), suggesting that PGC-1α deficiency reduced the formation of stable adhesions.

Fig. 1

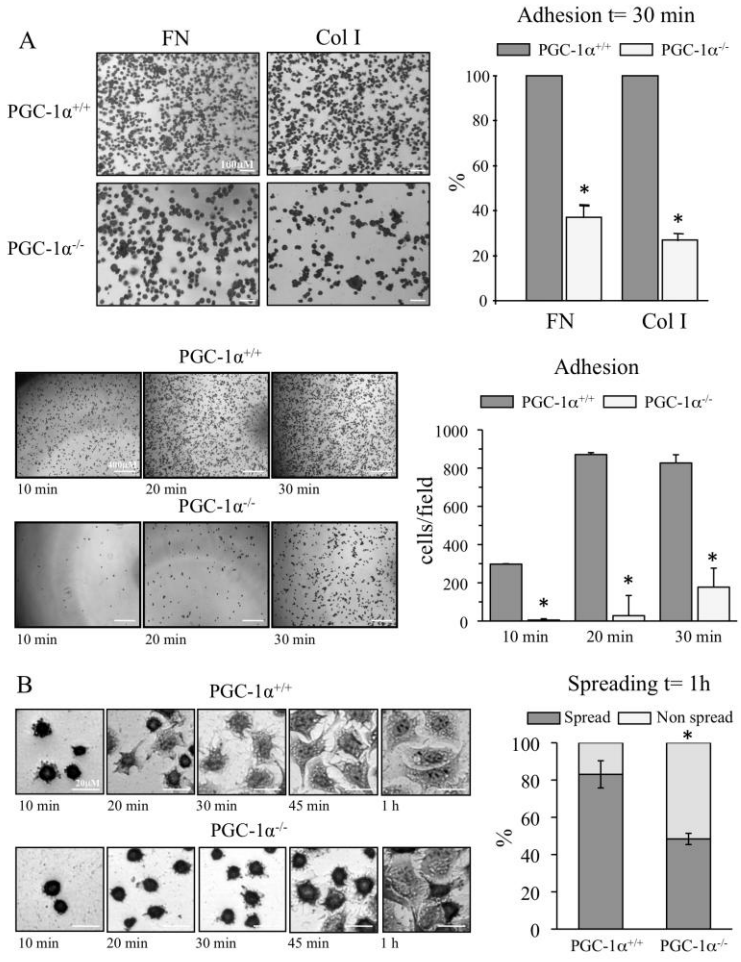

Figure 1. Endothelial cell adhesion and spreading dynamics are regulated by PGC-1α. Wild-type and PGC-1$\alpha^{-/-}$ MLECs were seeded on plates coated with fibronectin or collagen I and allowed to adhere for up to 30 min (**A**) or to spread for up to 2 h (**B**). Cells were washed fixed, bright field visualized on a microscope and counted. Left panels show images from representative experiments. Right panels show the quantifications. Data are means +SD (*) $p \leq 0.05$ vs. control. **A, top panel)** n=9. **A, bottom panel)** n=8. **B)** n=6.

Absence of PGC-1α results in unstable adhesion and spreading

To corroborate these results, we monitored the cell adhesion-spreading process using time-lapse video imaging of cell movements for 1 h after attachment to collagen I matrix. In contrast to wild-type MLECs, which displayed very limited motility after initial spreading, PGC-1$\alpha^{-/-}$ MLECs not only moved faster once attached, as has been previously described [14], but also showed incomplete and fully reversible spreading and detachment (Fig. 2A). Collectively, these results suggest that both the initial interaction with the matrix and also the formation of stable focal adhesions may be impaired in PGC-1$\alpha^{-/-}$ ECs.

To assess whether PGC-1α regulated the formation of focal adhesions, we next analyzed the focal adhesion complex-component paxillin. Paxillin is activated and phosphorylated by focal adhesion kinase (FAK) in response to integrin signaling, resulting in the formation of a focal adhesion complex. However, it must be dephosphorylated to allow the stabilization of the focal adhesion, and therefore can be used to monitor the turnover rate of newly formed focal adhesions. Analysis of phospo (p)-paxillin in MLECs that had been allowed to attach for 30 min, revealed that p-paxillin was localized to the tips of spreading filopoidia in wild-type MLECs (Fig. 2B). In contrast, PGC-1$\alpha^{-/-}$ MLECs displayed intense p-paxillin staining throughout the cell periphery, suggesting a random and highly active turnover of focal adhesions. These findings imply that the reduced adhesion capacity of PGC-1$\alpha^{-/-}$ MLECs is not due to an inability to form focal adhesion complexes but rather might be the consequence of an excessive FAK activity and high turnover rate [22]

Defective cytoskeleton dynamics in absence of PGC-1α

Cellular interactions with matrix components triggers stress fiber-formation of the polymerized actin cytoskeleton. In wild-type MLECs, F-actin was found concentrated in the extending filopodia of spreading cells 1 h after plating, and stress fibers were fully formed 2 h following plating (Fig 2B). In contrast, MLECs from PGC-1$\alpha^{-/-}$ mice exhibited dispersed F-actin foci throughout the cell periphery, and no stress fibers were detected even after 2 h (Fig 2B). To determine if this disorganization was a consequence of a deficient cell adhesion process, or could also be conspicuous in other situations, we analyzed the actin cytoskeleton of migrating and confluent cell cultures. Interestingly, while actin filaments reorganized in wild-type cells, and were about 2.5x more concentrated in the leading edge than in the retracting edge, this was barely detectable in PGC-1$\alpha^{-/-}$ MLECs (Fig. 2C). In confluent cultures, actin filaments are normally more concentrated in the cellular periphery than in the cytosol. Remarkably, this difference was more marked in PGC-1$\alpha^{-/-}$ MLEC, while mean cytosolic phalloiding stain was lower PGC-1$\alpha^{-/-}$ MLEC (Fig. 2C). These two characteristics could be related to the higher mobility of these cells. Moreover, PGC-1$\alpha^{-/-}$ MLECs were, on average, approximately 50% larger than wild-type counterparts, possibly a consequence of actin disorganization (Fig. 2D).

Endothelial cells show reduced formation of adherens junctions in absence of PGC-1α

To test whether intercellular junctions were also affected by the absence of PGC-1α, we analyzed VE-cadherin staining in confluent cultures. VE-cadherin plays a major role in the organization of intercellular adherens junctions in ECs. PGC-1$\alpha^{-/-}$ MLECs exhibited a reduction in VE-cadherin levels relative to wild-type cells; also, staining was not continuous and gaps could be detected together in zones where the membranes were not in direct contact despite the confluence of the culture (Fig. 2D, Supp. Fig. 1).

Fig. 2

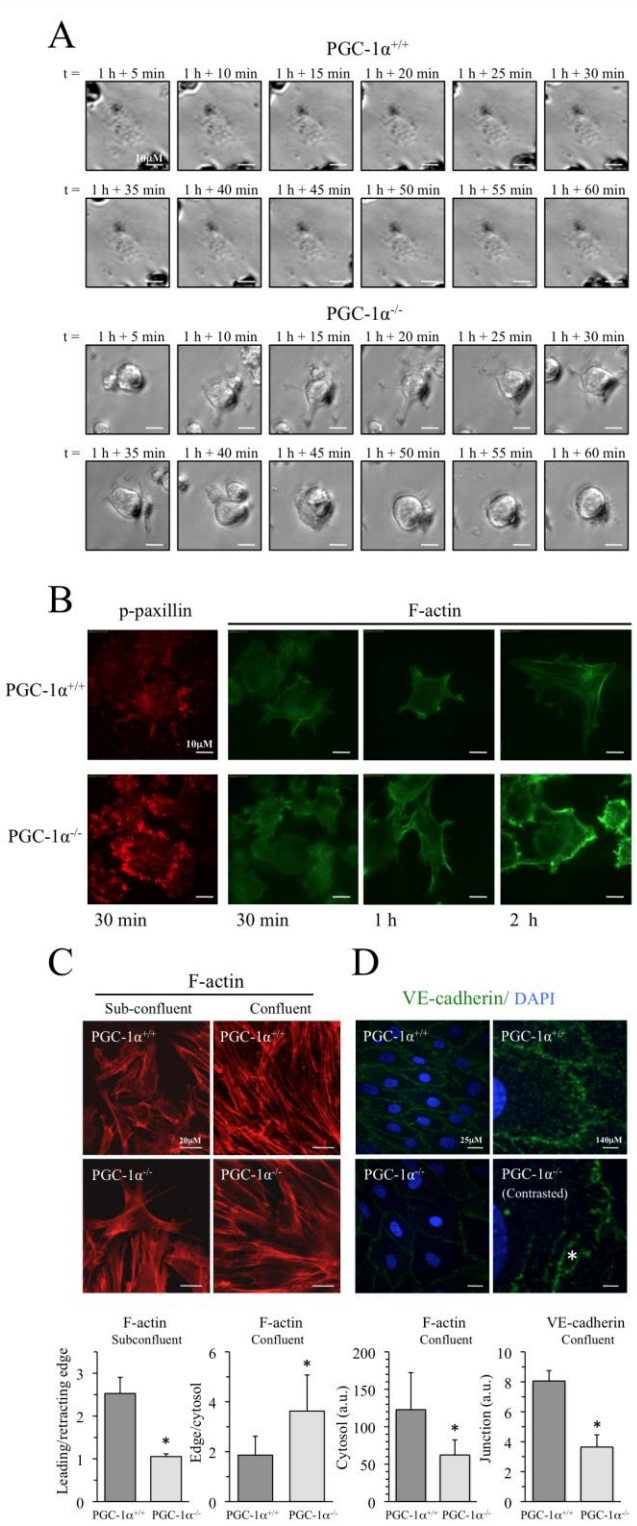

Figure 2. PGC-1α$^{-/-}$ MLEC form unstable cell-matrix and cell-cell contacts. A) Time-lapse bright field video images of live cells that have been allowed to adhere for 1 h. Images show the dynamics of representative cells at 5 min intervals for 1 h (n=3). **B)** Focal adhesion dynamics was analyzed by IF staining of cells with a p-paxillin antibody and polymerized actin filaments with phalloidin. Focal adhesions were visualized using a TIRF microscope (n=3). **C)** Wild-type and PGC-1α$^{-/-}$ MLECs actin filaments stained with phalloidin in sub-confluent and confluent MLECs and visualized with a fluorescence microscope (n=3). **D**) IF of VE-cadherin of confluent wild-type and PGC-1α$^{-/-}$ MLECs on a fibronectin-coated plate. Nuclei were stained with DAPI (n=3). Data are means +SD (*) $p \leq 0.05$ vs. control.

PGC-1α regulates endothelial cell coordination during migration

To assess how these deficiencies could impact on cell migration processes, we monitored cell motility using *in vitro* scratch assays and time-lapse video imaging. Whereas wild-type cells appeared to stream together as they migrated, we noted that the absence of PGC-1α resulted not only in an accelerated cell movement as already described [14], but also on a pronounced tendency to disperse over a larger area (Fig. 3A, lower panel; Supp. Info. Video Files). A similar effect was observed in BAECs upon PGC-1α silencing (Fig. 3A, lower panel; Supp. Info. Video Files). The Figure 3A shows only one side of the scrach in order to increase the magnification of the image and therefore be able to visualize how the cells spread out into the gap. In the images can be appreciated that cell density is lower when PGC-1α is absent or reduced while it is higher in over-expressing cells. This is not due to differences in confluency, all cultures were 100% confluent, but is mainly attributable to the bigger size (about 50%) of PGC-1α deficient cells (Fig. 2D). Nevertheless, PGC-1$\alpha^{-/-}$ fully-confuent cultures always show small gaps between cells (Fig. 2D), these could be attribute to problems in the formation of cell-cell contacts.

Fig. 3

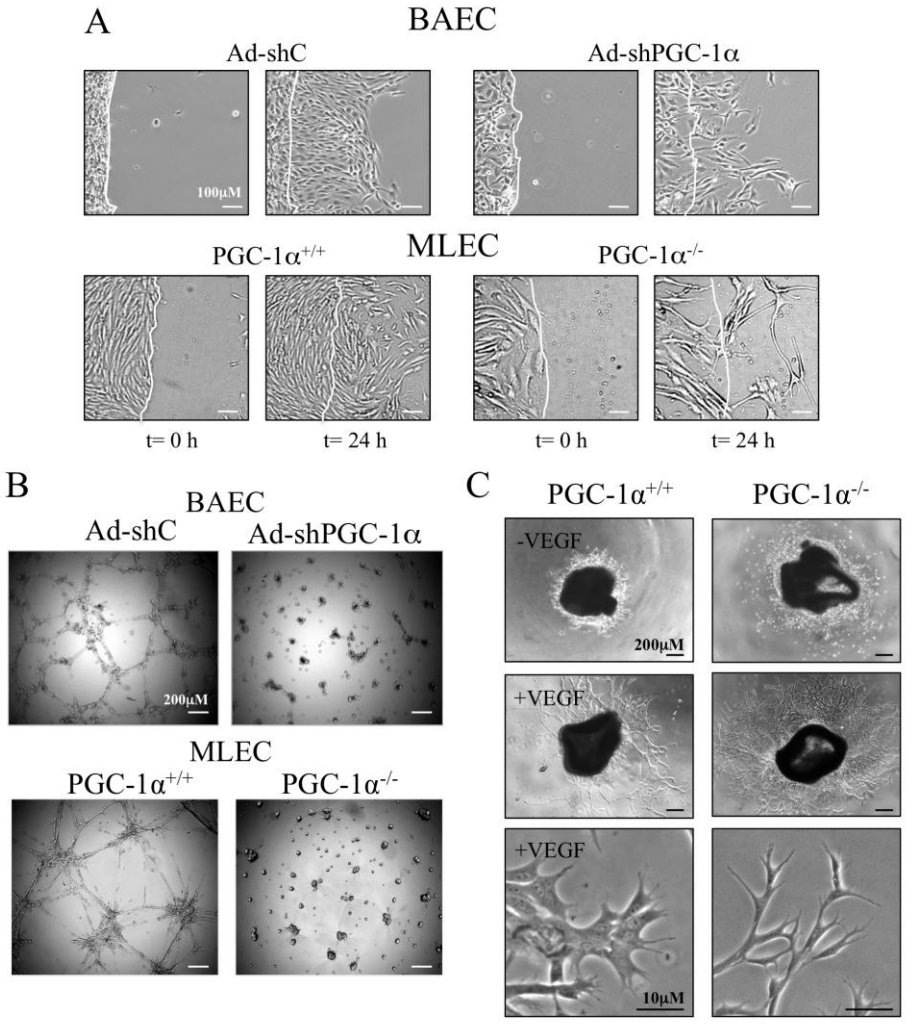

Figure 3. Lack of PGC-1α results in reduced intercellular junction/coordination during migration, and failure to form angiotubes *in vitro*. A) Scratch assays of BAECs infected with the indicated adenovirus to knock-down PGC-1α, and wild-type or PGC-1$\alpha^{-/-}$ MLECs. The migratory front is highlighted with a white line (n=6). **B)** *In vitro* angiotube assay on Matrigel using BAECs silenced for PGC-1α (upper panel), and wild-type and PGC-1$\alpha^{-/-}$ MLECs (lower panel) (n=5). **C)** Endothelial cells sprouting from aorta explants from wild-tpe and PGC-1$\alpha^{-/-}$ mice. Bright field images were taken after 5 days, the lower panels show higher magnification images of tip cells from the migratory edge (n=6). Data are means +SD (*) $p \leq 0.05$ vs. control.

Absence of PGC-1α results in impaired angiotube formation

The ability to form stable cell-to-cell contacts is not only important to ensure organized cell migration, it is also critical for the formation and stabilization of the newly formed vessel. Thus, we next tested vessel formation using an *in vitro* angiotube formation assay. Interestingly, results from silencing of PGC-1α expression in BAECs (Fig. 3B upper panel) and the absence of PGC-1α in MLECs (Fig. 3B lower panel), showed that both approaches led to an almost complete inability of ECs to form angiotubes *in vitro*, even though cells migrated and aggregated. This result suggested that deficient interactions of intercellular junctions provoked by the absence of PGC-1α are likely to be crucial for *in vivo* formation of blood vessels.

PGC-1α$^{-/-}$ endothelial cells display an enhanced tip phenotype

In a complementary approach to test EC migration and intercellular junction interactions in combination, we utilized an *ex vivo* angiogenesis assay using Matrigel-embedded aortic ring explants. We found that even in the absence of VEGF-A, PGC-1α$^{-/-}$ ECs had the capacity to migrate, while wild-type ECs could not migrate unless VEGF-A was added to the medium, and then did so in an individual manner, in the absence of interacting cells (Fig. 3C, upper panel). Moreover, we observed that, in the presence of VEGF-A, the cellular "chords" of sprouting cells exiting the aortic rings from PGC-1α$^{-/-}$ mice appeared thinner than those sprouting from wild-type aortic rings. Upon closer inspection of migratory edges, we observed that while wild-type ECs where closely packed, in several cell-wide chords, with filopodia only in the outermost migratory edge, PGC-1α$^{-/-}$ cells formed single-cell chords, and were highly elongated with extensive filopodia (Fig. 3C, lower panel). These observations suggested that in the absence of PGC-1α endothelial cells can migrate even in the absence of VEGF and they do not respond normally to VEGF-A, possibly inducing an enhanced tip-like phenotype.

PGC-1α$^{-/-}$ endothelial cells respond to antioxidant treatment enhancing VEGF-A signaling

Our previous *in vitro* results indicated that PGC-1α$^{-/-}$ MLECs exhibited a greater migration capacity compared with wild-type cells due to an overproduction of hydrogen peroxide [14]. Indeed, hydrogen peroxide is acknowledged as an important player in angiogenesis [18] and mediate EC migration and proliferation [23]. Thus, we evaluated whether the angiogenesis defects found in PGC-1α$^{-/-}$ mice could be attributed to an excess production of hydrogen peroxide. To this end, we treated PGC-1α$^{-/-}$ MLECs with an antioxidant and measured their ability to form angiotubes *in vitro*. Pre-incubation of PGC-1α$^{-/-}$ MLECs with EUK-189, a synthetic superoxide dismutase/catalase mimetic, enhanced the formation of angiotubes (Fig. 4, Supp. Fig. 2), pointing to a participation of hydrogen peroxide in capillary formation. To elucidate the molecular mechanisms that could link elevated hydrogen peroxide levels with the vascular instability observed in PGC-1α$^{-/-}$ mice, we next analyzed the response of wild-type and PGC-1α$^{-/-}$ MLECs to VEGF-A. This signaling pathway has been suggested previously to be highly sensitive to hydrogen peroxide [17, 18]. Consistently, PGC-1α$^{-/-}$ MLEC showed increased levels of phosphorylated VEGFR2, phosphorylated AKT and greater levels of NICD, compared with wild-type cells in the absence of VEGF-A (Fig. 5). Furthermore, relative to wild-type cells, the response to VEGF-A treatment was very poor in PGC-1α$^{-/-}$ MLECs in terms of VEGFR-2 induced phosphorylation, supporting the notion that PGC-1α-deleted ECs are largely insensitive to VEGF-A activation. Importantly, when PGC-1α$^{-/-}$ MLECs were pre-incubated with EUK-189, the response to VEGF-A was partially recovered, while the basal levels of phosphorylated VEGFR2, phosphorylated AKT and NICD, were reduced (Fig. 5). This observation is consistent with previous reports demonstrating the sensitivity of VEGFR-2 and its phosphatases to hydrogen peroxide [17, 18]. Moreover, these findings indicate that the response of VEGFR2 to VEGF-A requires the tight control of intracellular hydrogen peroxide levels. This control may be lost when important metabolic regulators such as PGC-1α are largely inactive [3]. mRNA levels where not significantly affected by EU-189 (Suppl. Fig. 3).

Fig. 4

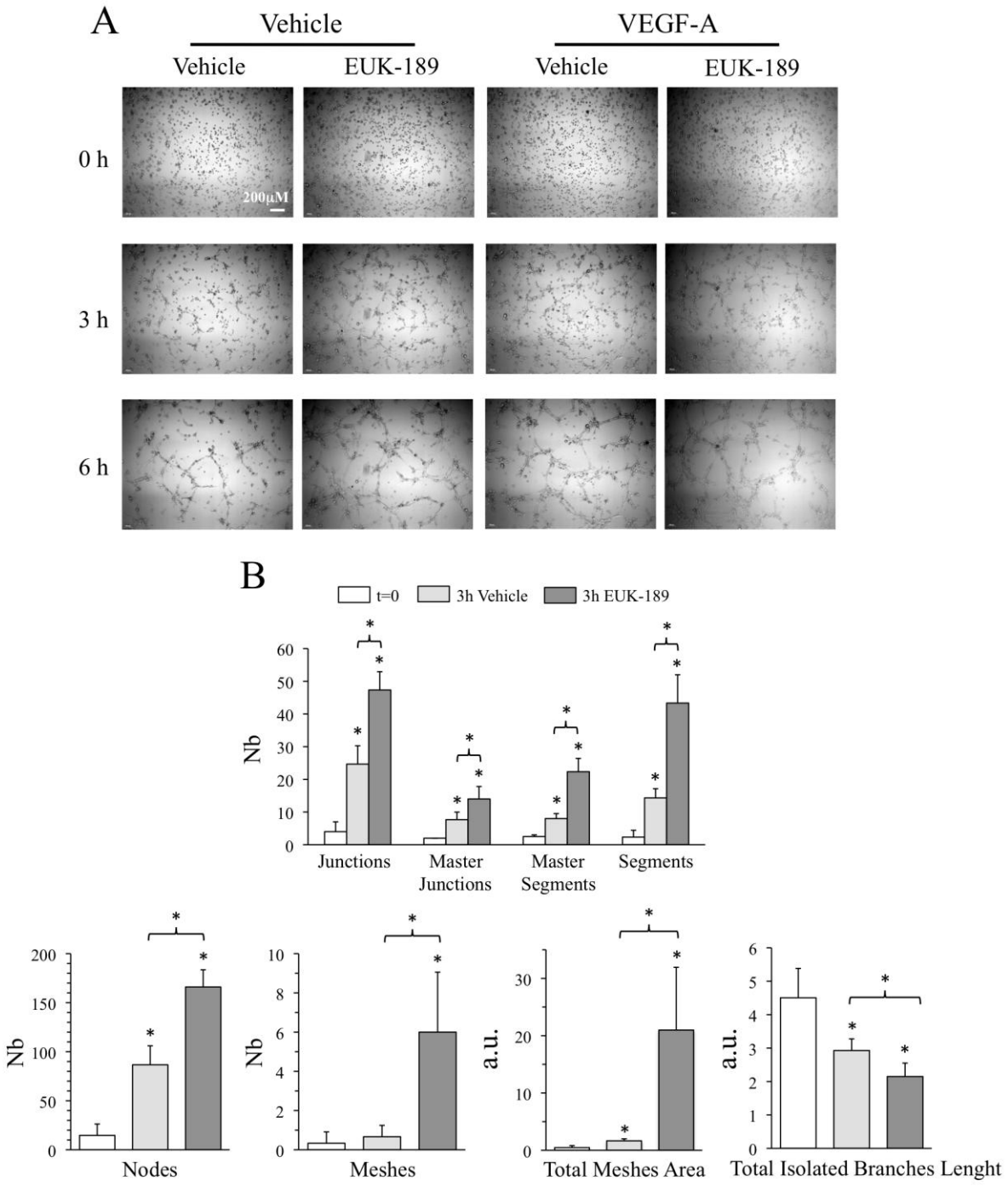

Figure 4. Antioxidant treatment improves *in vitro* angiotube formation in PGC-1α$^{-/-}$ MLECs. A) *In vitro* angiotube assay on Matrigel using PGC-1α$^{-/-}$ MLECs, treated with VEGF and/or EUK-189 (n=3). **B)** Analysis of structures formed in the angitube assay. Data are means +SD (*) $p \leq 0.05$ vs. control.

Fig. 5

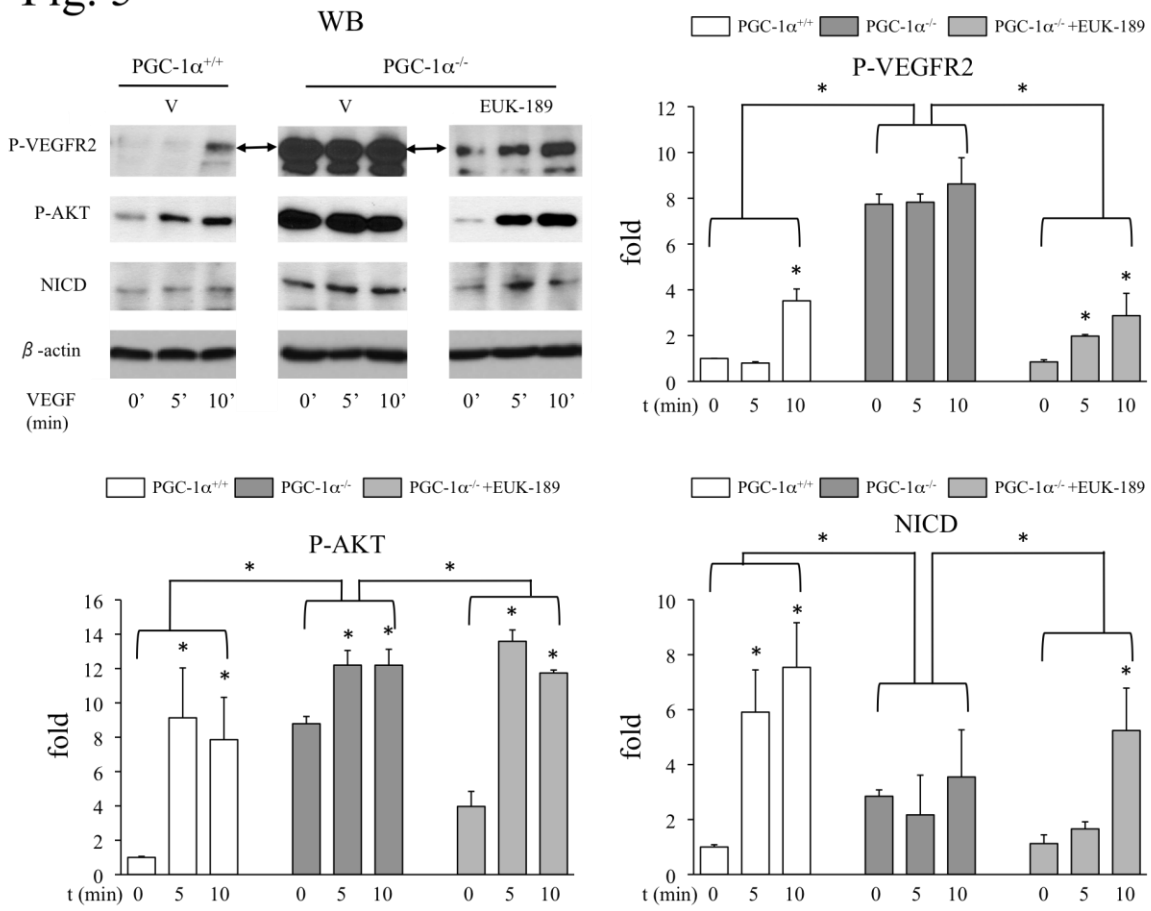

Figure 5. Antioxidant treatment restores VEGF signaling in PGC-1α$^{-/-}$ MLECs. PGC-1α$^{-/-}$ MLECs where treated with VEGF and/or EUK-189 for the indicated time periods. Western blot analysis of whole cell extracts of relevant proteins in VEGF signaling: VEGFR2, NICD and AKT (n=3). Data are means ±SD (*) $p \leq 0.05$ vs. control.

PGC-1α has been shown to induce the expression of several mitochondrial antioxidant proteins, and its absence results in enhanced mitochondrial superoxide levels. Concomitantly, PGC-1α also regulates the expression of important extra-mitochondrial antioxidants, like catalase. In order to validate the contribution of mitochondrial ROS to the observed alteration in VEGF signaling, we treated PGC-1α$^{-/-}$ MLEC with a mitochondria-targeted SOD mimetic, MitoTEMPO and evaluated the effect on VEGF-A signaling. We found a significant reduction in the basal levels of phosphorylation of VEGFR2 and AKT, as well as a reduction in the basal levels of NICD (Fig. 6A, central panel). VEGF-A treatment also resulted in an enhanced fold induction of VEGFR2 and AKT phosphorylation, as well as in the levels of NICD upon stimulation (Fig. 6A, bottom panel). These results suggest that there is a relevant contribution of mitochondrial ROS production to the observed phenotype.

Finally, since hydrogen peroxide dependent inactivation of VEGF signaling pathway has been proposed to be mediated by NOX activity, we tested whether NOX inhibition would be able to restore VEGF-A signaling in PGC-1α$^{-/-}$ cells, and we found that pretreatment with the general NOX inhibitor VAS-2870 also resulted in significant reduction in the basal levels of phosphorylation of VEGFR2 and AKT and an enhanced sensitivity (fold induction) of the VEGFR2 receptor to VEGF-A dependent phosphorylation, that correlated with and enhanced AKT phosphorylation, supporting the involvement of NOX activity (Fig. 6B).

Fig. 6

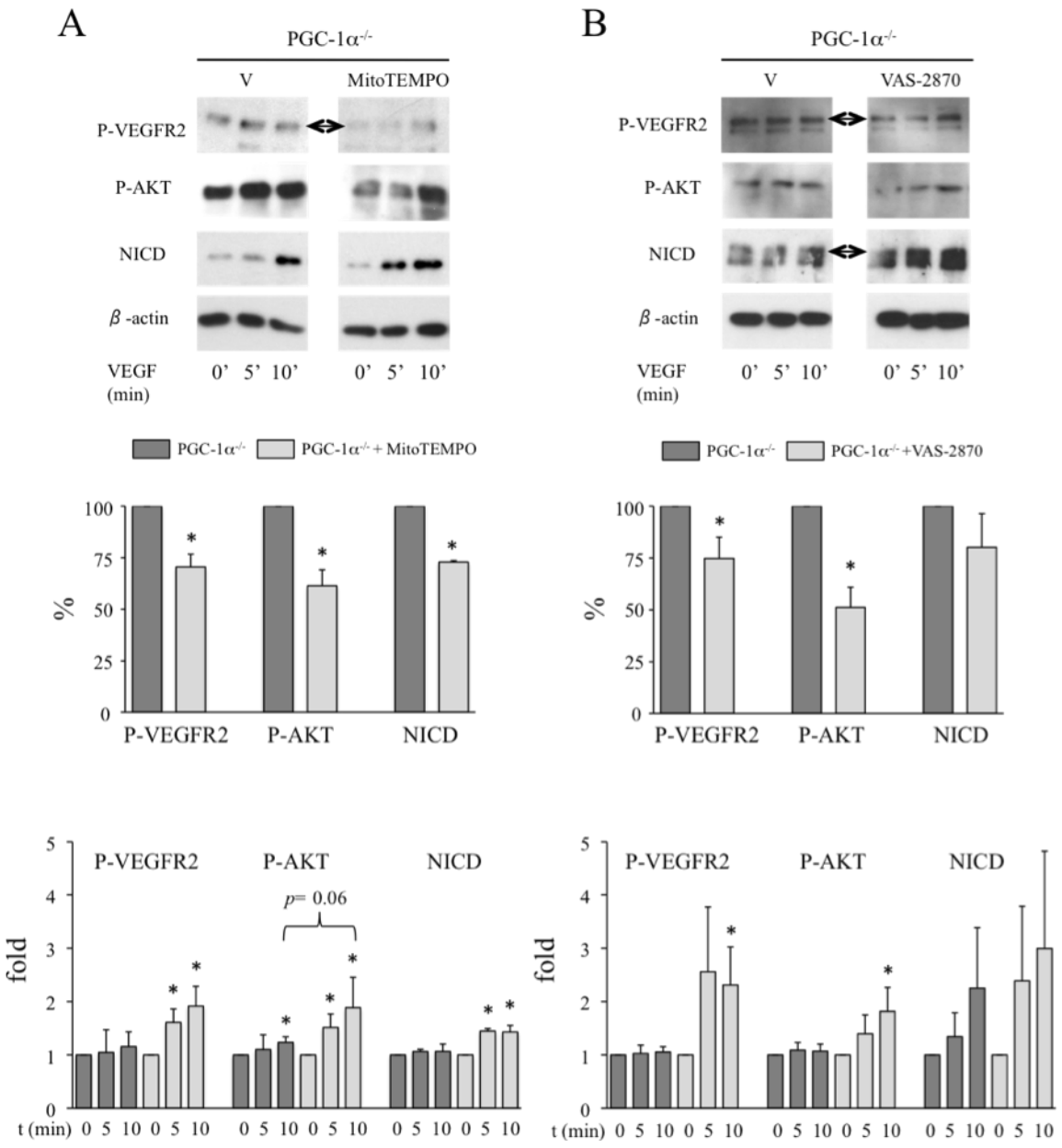

Figure 6. Both detoxification of mitochondrial ROS and NOX inhibition restores VEGF signaling in PGC-1α$^{-/-}$ MLECs. PGC-1α$^{-/-}$ MLECs where pre-treated with the mitochondria-targeted SOD mimetic MitoTEMPO (10 μM) for 3 h **(A,** n=5**)** or with a general NOX inhibitor (VAS-2870, 20 μM) for 2h (**B,** n=3), then VEGF-A was added and downstream signaling (VEGFR2, NICD and AKT) was analyzed by western blot (n=5). Top panels show representative western blots. Central panels show the quantitative analysis of data at time 0, prior to VEGF-A addition. The bottom panels show the fold induction upon VEGF-A addition, values are normalized for those at time 0, that have been assigned the arbitrary value of 1. Data are means ±SD (*) $p \leq 0.05$ vs. control.

Discussion

The results of this study show that regulation of ROS homeostasis by PGC-1α plays an important role in the control of endothelial function, in particular in the regulation by VEGF-A of the stability of the cellular and cellular-matrix contacts. We demonstrate that, in the absence of PGC-1α, the vascular endothelial cells appears to be constitutively activated, characterized by faster migrating ECs which adhere slowly to the substrate. Also, ECs spread slowly and can reverse this process and detach again. These alterations are likely due to the low stability and high turnover of the focal adhesions, an inability to form a structured f-actin cytoskeleton, and a reduced formation of VE-cadherin adherens junctions. Succinctly, ECs interact poorly with the substrate and with other cells. Clearly, these alterations are likely to be relevant in metabolic disorders where PGC-1α activity is generally found to be low, and in particular might be related to the development of diabetic retinopathy.

Reduced intercellular contacts in PGC-1α-deficient ECs resulted in the loss of coordinated cell movement in scratch assays, and a failure to form angiotubes *in vitro*. Analysis of the endothelial sprouting process from aortic rings further suggested that PGC-1α-deficient cells actively migrate even in the absence of VEGF-A, and form tip cells independently of their location, indicating a defective response to VEGF-A. Treatment of MLECs with VEGF-A *in vitro,* together with analysis of the VEGF pathway, confirmed that the absence of PGC-1α resulted in the constitutive activation of the VEGF-A signaling pathway, plus a poor response to exogenous VEGF-A. This defective response to VEGF-A may explain the constitutively-activated phenotype of PGC-1α deficient ECs.

PGC-1α deficient ECs have elevated levels of oxygen superoxide and hydrogen peroxide [5]. Given that the VEGF-A signaling pathway has been shown to be sensitive to hydrogen peroxide, and that oxidative modifications of VEGFR2 [18, 24] and its main phosphatases have been described [17], we hypothesized that elevated ROS levels found in PGC-1α-deficient ECs would be responsible for the poor VEGF-A response. Consistent with this idea, we found that antioxidant treatment largely restored normal VEGF-A response in PGC-1α deficient ECs. Furthermore, EUK-189 treatment also restored the capacity of those cells to form angiotubes *in vitro*, indicating a normalization of the inter-cellular interactions.

Long regarded as by-products of oxidative metabolism, ROS are now recognized as signaling mediators in their own right. The prototypic redox-regulated targets are the protein tyrosine phosphatases that are inactivated by hydrogen peroxide; however, identification of redox-sensitive kinases such as Src has led to the slow emergence of a role for redox regulation of tyrosine kinases by concurrent oxidative activation of tyrosine kinases. Thus, ROS fine-tune the duration and amplification of the phosphorylation signal. Importantly, the activity of VEGFR2 and two of its phosphatases, DEP1 and PTP1B, have been shown to be regulated by hydrogen peroxide [17] [24] [18]. Demonstration of the physiological relevance of these regulations has been hampered by the lack of relevant disease models, since the majority of experimental evidence derives from *in vitro* assays with artificially generated or exogenously added ROS; this is the case for VEGFR-2 signaling pathways.

PGC-1α is known to regulate the levels of mitochondrial ROS [5]. However, most recent studies stress the role played by NADPH oxidases (NOXs) in the ROS-dependent control of growth factor signaling processes [25] and less attention has been paid to the role of mitochondrial ROS. Nevertheless, although a largely neglected area of research, a body of evidence shows that elevated mitochondrial production of ROS is commonly found in cells with elevated NOX activity, and it has been shown that mitochondria can regulate NOX function and *viceversa* [26]. Additionally, most, if not all, physiological settings where ROS appear to play an important role, including angiogenesis, describe both enhanced NOX and elevated mitochondrial ROS levels [27, 28]. Furthermore, mitochondria-targeted antioxidants have been proposed to have potential therapeutic value in retinopathy [29]. Nonetheless, it remains to be determined whether PGC-1α could also directly regulate the expression or activity of NOX or alternatively of PrxII, the peroxiredoxin antioxidant that appears to modulate

VEGFR2 oxidation [18].

In conclusion, our study strongly supports the idea that PGC-1α control of hydrogen peroxide levels in endothelial cells plays a major role in the formation of stable interactions of endothelial cells with other cells and with the matrix, a process likely to be relevant in metabolic disorders where microvascular complications are prevalent, like diabetic retinopathy. Since antioxidant therapies have shown significant caveats, alternative therapies that aim to re-wire normal ROS homeostasis, boosting PGC-1α activity might represent a therapeutic approach for retinopathy patients.

Acknowledgements

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Conflict of Interest

None declared.

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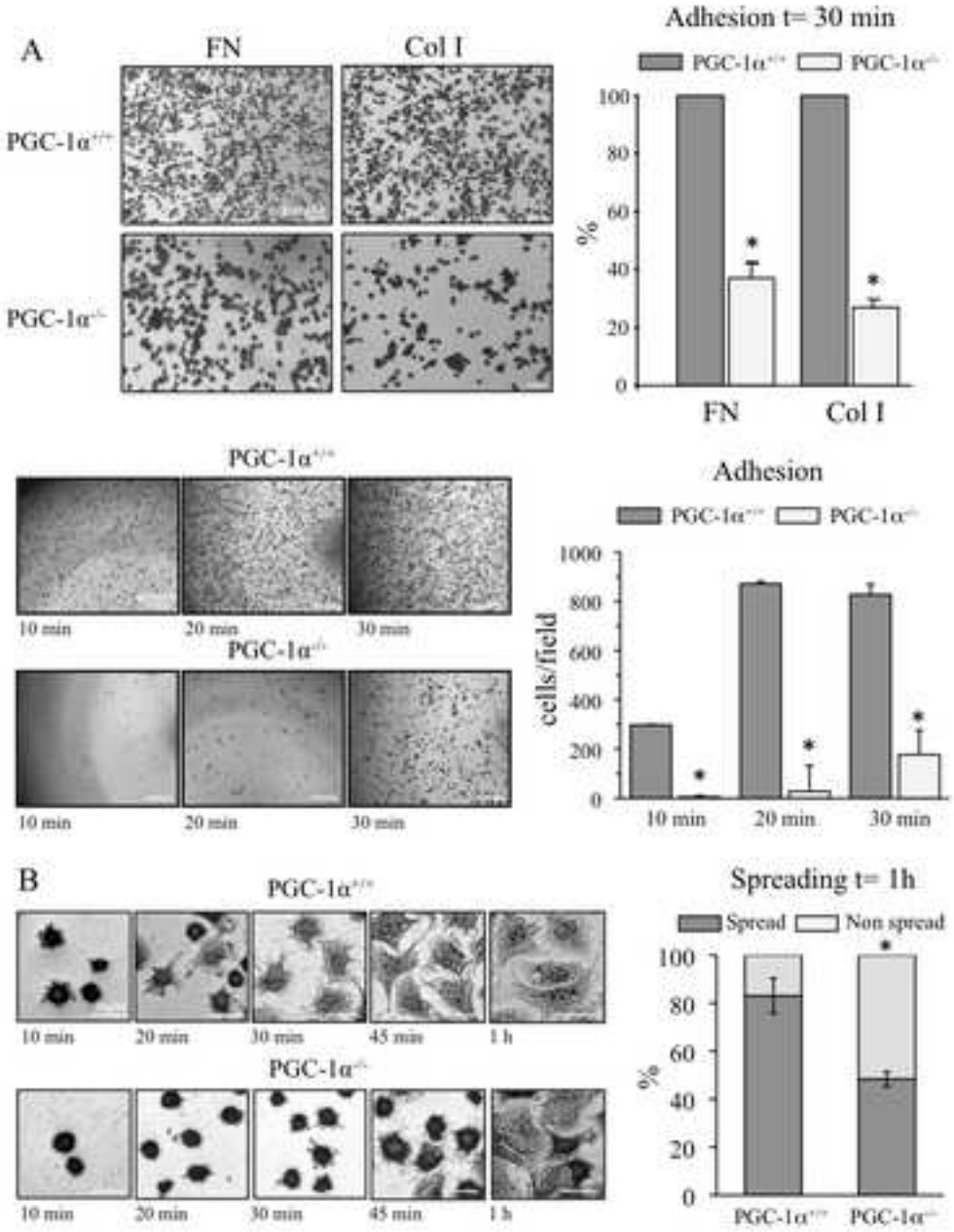

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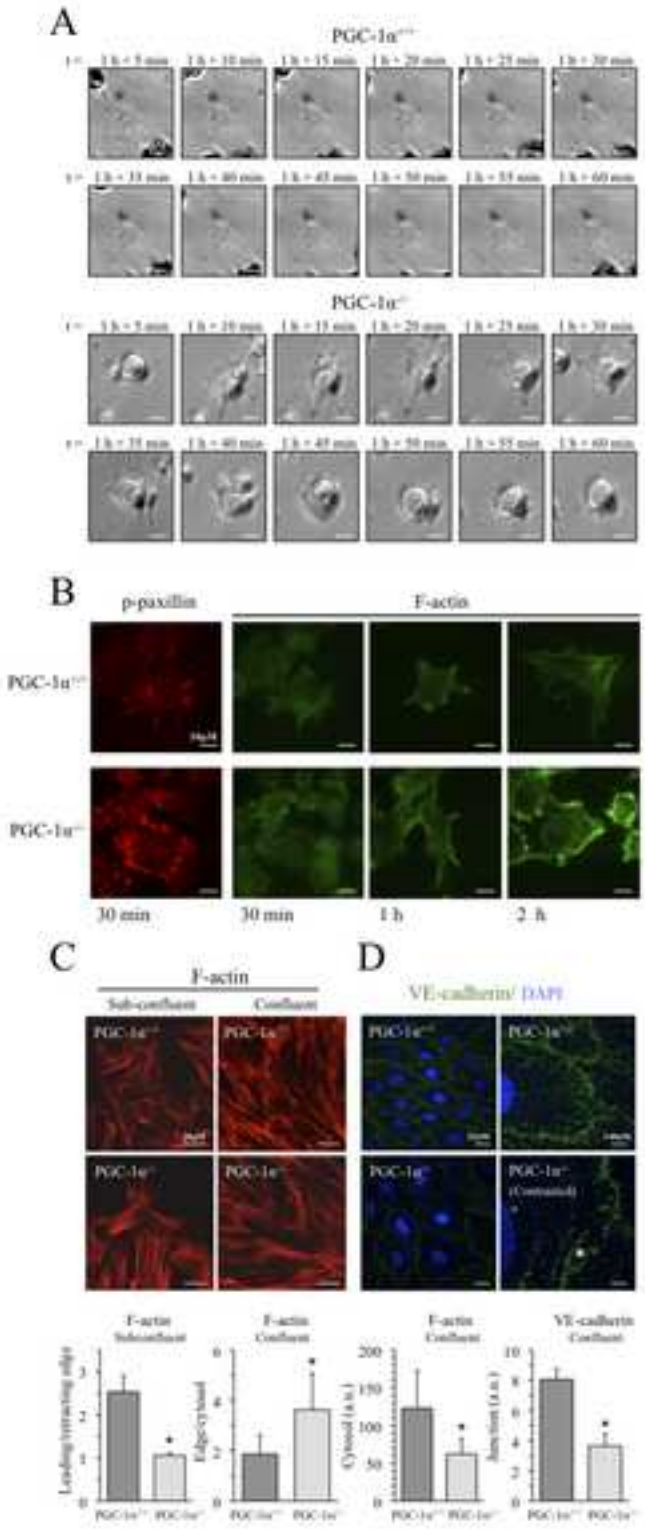

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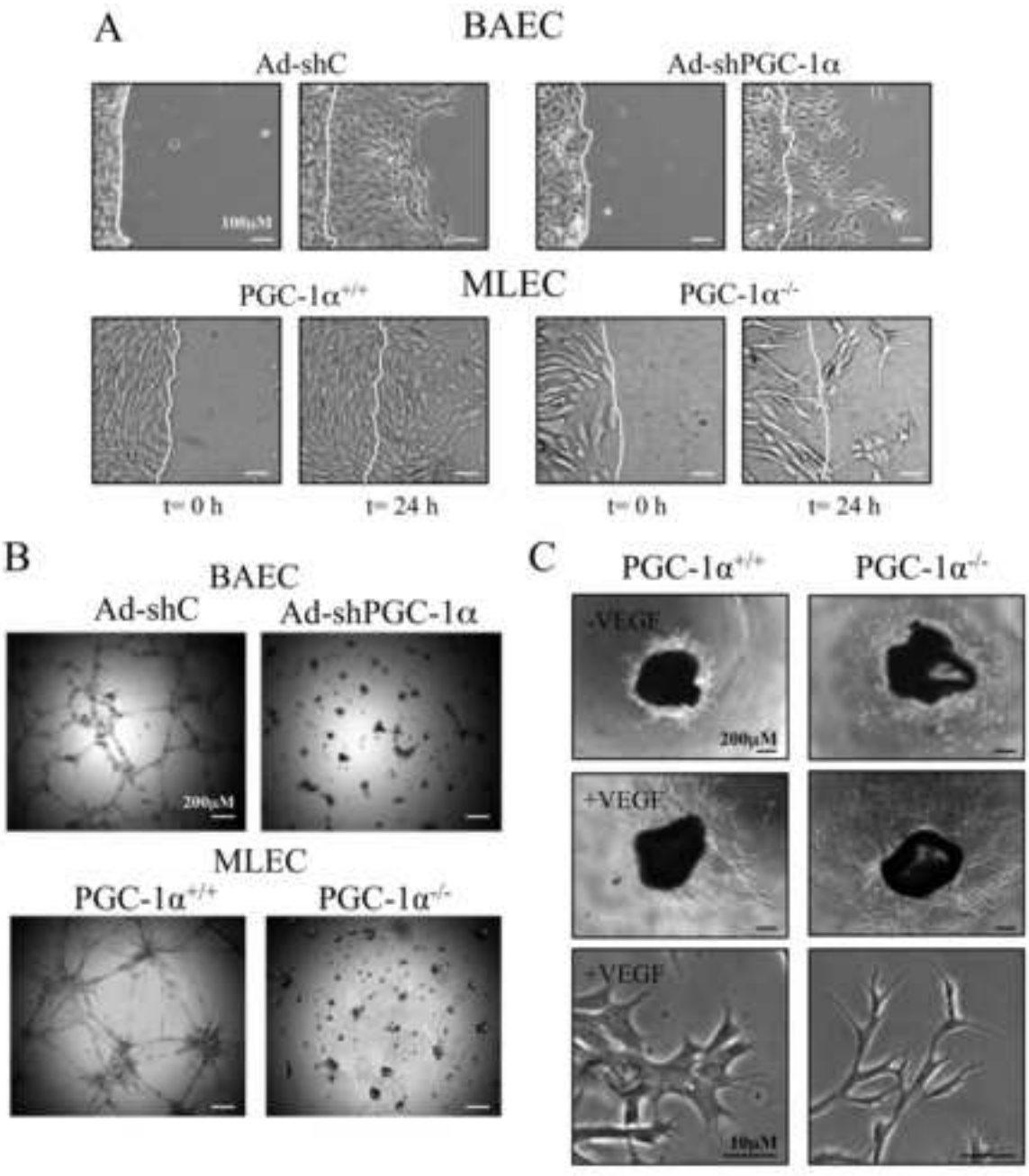

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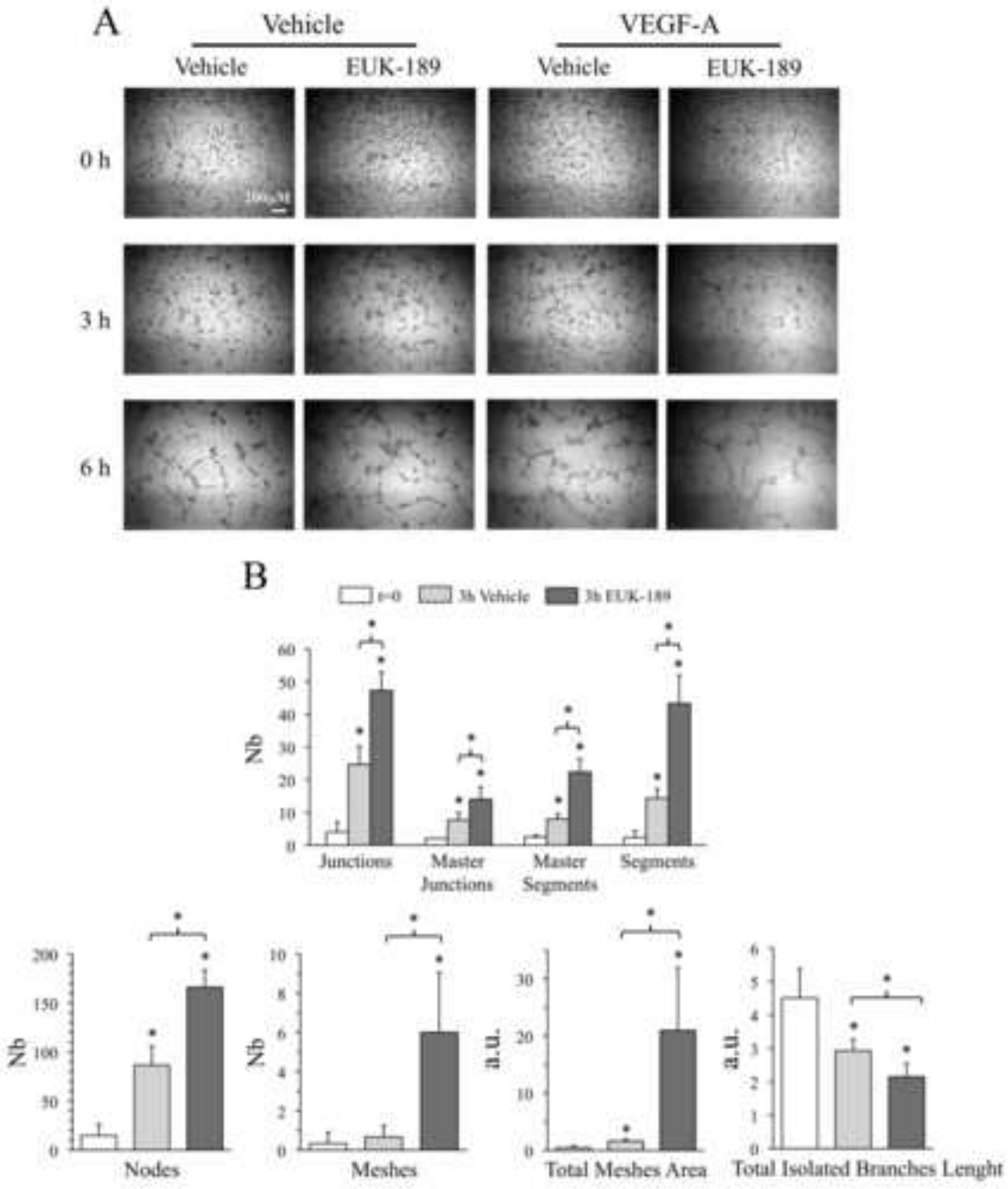

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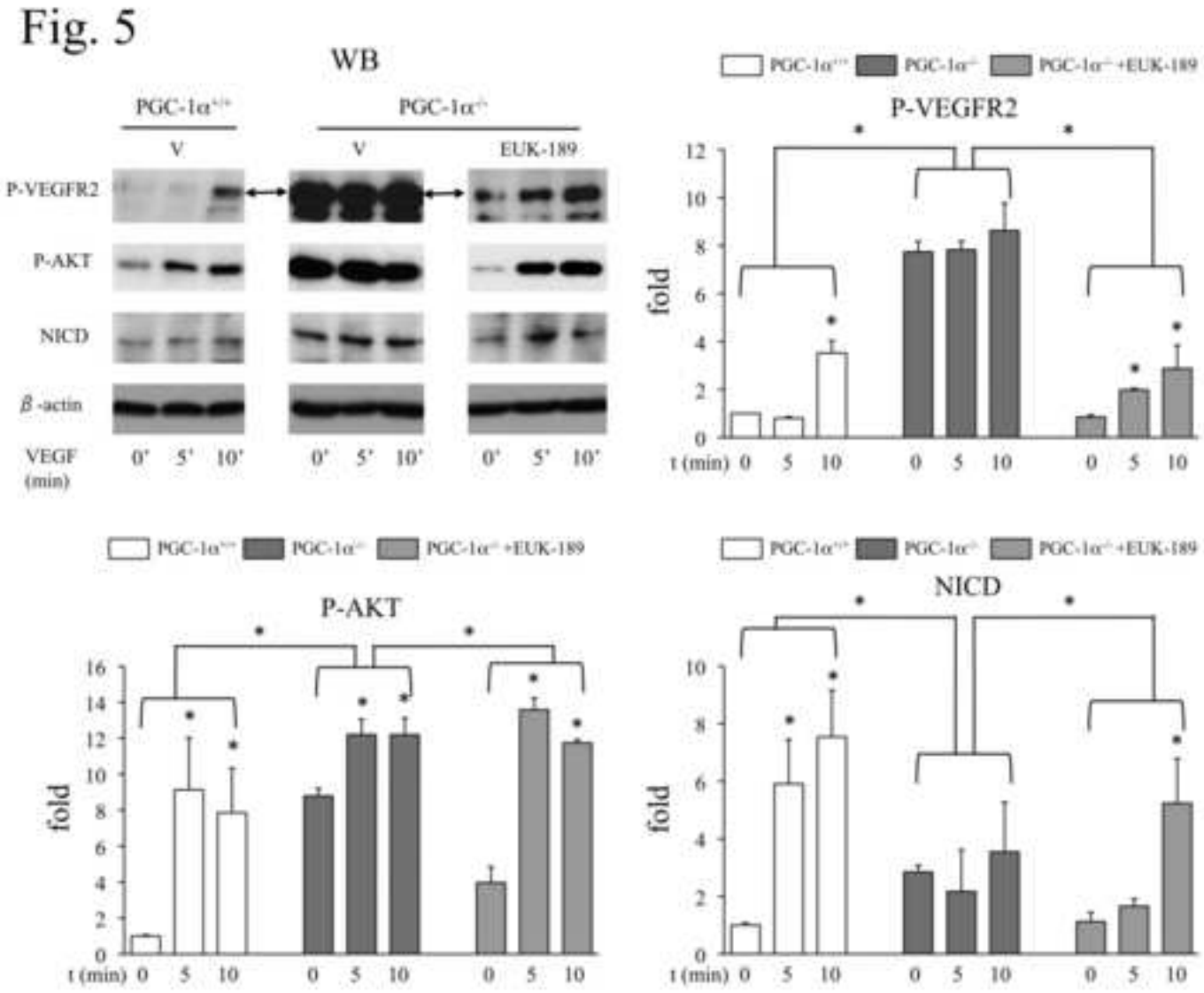

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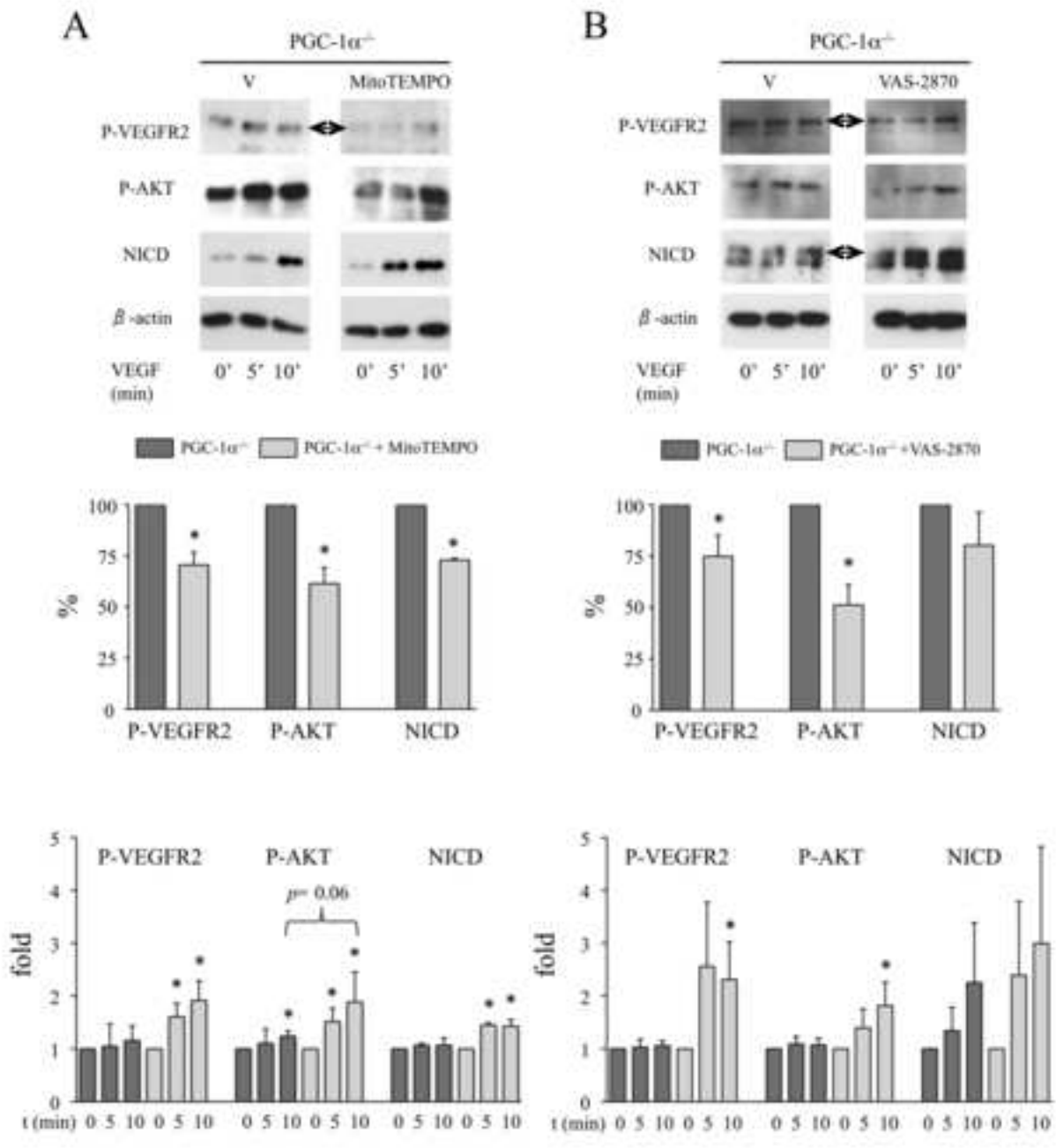

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