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Transcriptional and Functional Adaptations of Human Endothelial Cells to Physiological Chronic Low Oxygen[1]

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ABSTRACT

Endothelial cells chronically reside in low-O_2 environments in vivo (2%–13% O_2), which are believed to be critical for cell homeostasis. To elucidate the roles of this physiological chronic normoxia in human endothelial cells, we examined transcriptomes of human umbilical vein endothelial cells (HUVECs), proliferation and migration of HUVECs in response to fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA), and underlying signaling mechanisms under physiological chronic normoxia. Immediately after isolation, HUVECs were cultured steadily under standard cell culture normoxia (SCN; 21% O_2) or physiological chronic normoxia (PCN; 3% O_2) up to 25 days. We found that PCN up-regulated 41 genes and down-regulated 21 genes, 90% of which differed from those previously reported from HUVECs cultured under SCN and exposed to acute low O_2. Gene ontology analysis indicated that PCN-regulated genes were highly related to cell proliferation and migration, consistent with the results from benchtop assays that showed that PCN significantly enhanced FGF2- and VEGFA-stimulated cell proliferation and migration. Interestingly, pre-exposing the PCN cells to 21% O_2 up to 5 days did not completely diminish PCN-enhanced cell proliferation and migration. These PCN-enhanced cell proliferations and migrations were mediated via augmented activation of MEK1/MEK2/ERK1/ERK2 and/or PI3K/AKT1. Importantly, these PCN-enhanced cellular responses were associated with an increase in activation of VEGFR2 but not FGFR1, without altering their expression. Thus, PCN programs endothelial cells to undergo dramatic changes in transcriptomes and sensitizes cellular proliferative and migratory responses to FGF2 and VEGFA. These PCN cells may offer a unique endothelial model, more closely mimicking the in vivo states.

angiogenesis, growth factors, physiological chronic low oxygen, protein kinases, transcriptome

INTRODUCTION

Endothelial cells physiologically reside in low-O_2 environments (~2%–13% O_2 or 15–100 mmHg pO_2) relative to atmospheric O_2 (~21% O_2 at sea level) in vivo [1–4]. In human placentas, the vasculogenesis and angiogenesis occur under low O_2 throughout pregnancy (~2% O_2 at ≤8–10 wk of gestation, ~8% O_2 between 8–10 wk, and ~6% O_2 at the end of the third trimester) [3, 4]. The O_2 level in the human fetus is even lower, ~3.7% O_2 (range, 2.3%–5.1%) in the umbilical vein at the end of gestation [4]. This physiological chronic low O_2 is believed to be critical for maintaining normal placental homeostasis and functions because alteration of O_2 tension can significantly affect placental development, including vascular growth and development [1–5]. For example, at high altitudes, where O_2 tension is lower than at sea level, placental villous vascularization is greatly increased in European pregnant women compared with those at sea level [6, 7]. Consistently, a growing body of evidence has also shown that more severe low O_2 is intimately associated with endothelial dysfunction, which is a hallmark for many vascular diseases, such as hypertension, pulmonary artery hypertension, preeclampsia, and cancer [1–5]. However, most of our current knowledge regarding endothelial behaviors and the underlying cellular and molecular mechanisms is built on endothelial models established under atmospheric O_2, which is actually extremely hyperoxic to most cells. These cells therefore most likely represent a population of cells specifically adapted to the hyperoxic condition, not closely mimicking in vivo states. Thus, investigating endothelial behaviors and underlying mechanisms under physiological chronic low O_2 will advance our understanding of normal endothelial function and the endothelial dysfunction occurring in many cardiovascular diseases related to endothelial dysfunction.

In addition to O_2, endothelial cells are also tightly regulated by peptide growth factors, including fibroblast growth factor 2 (FGF2) and vascular endothelial growth factor A (VEGFA), both of which are key regulators of placental angiogenesis and vasodilation [8–10]. The cellular responses to FGF2 and VEGFA are mediated by activating their high-affinity receptors. Upon activation, FGF2 and VEGFA receptors induce activation of a cascade of protein kinases, including mitogen-activated protein kinase 3/1 (also termed ERK1/2) and v-akt murine thymoma viral oncogene homolog 1 (AKT1), leading to cellular responses [8–10]. Numerous studies have revealed

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that endothelial preparations established under atmospheric O_2 exhibit enhanced angiogenic responses (e.g., endothelial proliferation and migration) after an acute exposure to low O_2 (~2%–5% O_2; 4–120 h) [11–14]. These stimulatory effects are thought to be mediated primarily via increasing expression of growth factors and their receptors or other modulators (e.g., metalloproteinases) [1, 2]. However, we have reported that acute exposure to low O_2 (3% O_2 for up to 48 h) fails to alter FGF2- and VEGFA-induced activation of ERK1/2 and AKT1, although such O_2 exposure promotes FGF2- and VEGFA-stimulated cell proliferation in human placental artery endothelial cells expanded under 21% O_2 [14], implying no participation of these two kinases in endothelial proliferation under such O_2 exposure.

Little is known regarding physiological chronic normoxia (2%–13% O_2) regulation of endothelial response to FGF2 and VEGFA, particularly in the aspect of underlying signaling mechanisms [15]. In the present study, we examined and compared the transcriptional profiles of human umbilical vein endothelial cells (HUVECs) cultured and expanded continuously under standard cell culture normoxia (21% O_2) and physiological chronic normoxia (3% O_2, which is within the physiological range of O_2 in the umbilical vein at the end of pregnancy [4], for ~20–25 days) using the microarray analysis. We also examined the proliferation and migration of HUVECs in response to FGF2 and VEGFA as well as the involvement of mitogen-activated protein kinase kinase 1/2 (also termed MEK1/MEK2)/ERK1/ERK2 and phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha (PI3K)/AKT1 pathways in mediating these cell responses.

MATERIALS AND METHODS

Additional methods section is available in the online supplement (all Supplemental Data are available online at www.biolreprod.org).

Endothelial Cultures

Human umbilical cords from normal term pregnancy patients with no complications were collected immediately after delivery. The cord collection protocol was approved by the Institutional Review Board of Meriter Hospital, and the Health Sciences Institutional Review Boards of the University of Wisconsin–Madison.

Human umbilical vein endothelial cells (HUVECs) were isolated using a standard collagenase enzyme digestion [14]. Immediately after isolation, cells obtained from the same vein were split equally, cultured, and expanded steadily under standard cell culture normoxia (37°C, 5% CO_2, and 95% air; designated as SCN) or physiological chronic normoxia (37°C, 5% CO_2, 3% O_2, and 92% N_2; designated as PCN) up to 25 days. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 100 μg/L heparin, and 37.5 mg/L endothelial cell growth supplement. For PCN culture and treatments, media were prepurged with N_2 and equilibrated in a hypoxia incubator before addition to cells. Dissolved O_2 in media was monitored using a dissolved oxygen meter. The PCN was also confirmed by determining cellular protein levels of hypoxia-inducible factor 1, alpha subunit (HIF1α), BCL2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3), and solute carrier family 2 (facilitated glucose transporter), member 3 (also termed glucose transporter 3 [GLUT3]).

After 3–4 days of culture, cells were sorted by flow cytometry based on their expression of platelet/endothelial cell adhesion molecule 1 (PECAM 1, also known as CD31; see Supplemental Methods). The purity of cells was also examined by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL; see Supplemental Methods). At passage 1, cells were subjected to the capillarylike tube formation assay to further confirm their endothelial phenotype. Only the cell preparations with ≥96% of cells exhibiting CD31 positivity and uptake of DiI-Ac-LDL, and being capable of forming capillarylike tube structures were used in this study (see Supplemental Methods). Cells at passage 3 (~15 days after isolation) were used for the microarray study, whereas cells at passages 4–5 (~20–25 days after isolation) were used for all other studies.

Paired SCN and PCN cell preparations, each of which was derived from the same vein, were used for all experiments conducted. All 3% O_2 experiments were performed in a heated oxygen-controlled glovebox (Coy Laboratory Products), and media were prepurged with N_2 and equilibrated to 3% O_2 before addition to cells.

Microarray Hybridization

Total RNA was extracted from six pairs of cell preparations of SCN and PCN HUVECs (total of 12) at 80% confluence using an RNeasy Mini Kit (Qiagen) in conjunction with a QIA shredder following the manufacturer's instructions [16]. Total RNA was quantified by the Nanodrop ND-100 spectrophotometer (Nanodrop Technologies). The microarray facility at the University of Wisconsin Biotech Center confirmed the quality and integrity of RNA using the Agilent RNA6000 NanoChip in the Agilent 2100 bioanalyzer (Agilent Technologies). Only high-quality RNA samples with an integrity number ~9.9 (in a range of 1–10, 10 is completely intact RNA) were subjected to further microarray analysis. Complementary RNA was synthesized, labeled, and fragmented using the MessageAmp II-biotin enhanced kit (Ambion). Fragmented cRNA (10 μg per sample) was hybridized to Affymetrix U133 plus 2.0 microarray chips (Affymetrix). The chips were processed on an Affymetrix GC450 Fluidics station. Data were extracted from GC3000 G7 scanned images using Affymetrix Expression Console software version 1.4. Microarray hybridization and postprocessing were carried out by experienced staff at the Biotech Center. To assess the quality of the entire assay and data analysis, routine quality controls were conducted according to standard Affymetrix protocols, which include visual array inspection, 3′/5′ glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin ratios, percentage of present calls, B2 signal, intensity of poly-A RNA and hybridization controls, and background and noise values. A scaling factor was used to correct variations between gene chips. The signal ratios of 3′ probe sets: 5′ probe sets of genes were calibrated by normalizing to the signal ratio of *GAPDH* and β-actin.

Microarray data were logged into the Gene Expression Omnibus at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo).

Microarray Data Analyses

The data were normalized with robust multiarray analysis. One outlier in the data set was identified using hierarchical clustering and eliminated from the following data analysis. EBarrays [17] was applied to identify differentially expressed (DE) genes. Specifically, a gene was identified as DE if its posterior probability of DE as assessed by EBarrays exceeded 0.99. This threshold was chosen to control the posterior expected false-discovery rate at 1%. A second filter was applied to ensure that the transcripts were expressed at a detectable level. In particular, an up-regulated gene was selected only if all of the probe sets in its corresponding condition were deemed ''present'' as characterized using the present/absent calls provided by Affymetrix postprocessing software (Microarray Suite version 5.0).

Hierarchical clustering of the microarray data was performed using the MeV with Pearson correlation [18]. The DE genes were then uploaded to the Ingenuity Pathway Analysis (IPA; Ingenuity) using a manually curated relationship from the literature.

Quantitative PCR

Quantitative PCR (qPCR) was conducted as described previously [16]. First-strand cDNA was synthesized by SuperScript II reverse transcriptase (Invitrogen) with T7-oligo(dT)24 primers. The qPCR was performed with SYBR Green I Master (Roche) and primers (Supplemental Table S1) in a Light Cycler 480 (Roche). All samples were run in triplicate. Negative controls (no template control and no enzyme control) were included in every set of amplification. The β-actin and TATA box-binding proteins selected by BestKeeper software [19] were used for normalization. RE-ST2005 software [20] was applied to determine the statistically significant difference and the relative fold change.

Western Blotting and Immunoprecipitation

Western blotting and immunoprecipitation were performed as described previously [16, 21, 22]. Placental tissues were homogenized and lysed by sonication in buffer (50 mM HEPES, 0.1 M NaCl, 10 mM ethylene diamine tetraacetic acid, 4 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM sodium orthovanadate [pH 7.5], 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 5 μg/ml leupeptin, and 5 μg/ml aprotinin). After centrifugation, protein concentrations of the supernatant were determined with bovine serum albumin (fraction V; Sigma) as standards. The protein samples (20 μg) were separated on SDS-PAGE gels and electrically transferred to polyvinylidene difluoride membranes. The membranes were immunoblotted with the antibody

against different targets (Supplemental Table S2). Proteins were visualized using enhanced chemiluminescence reagents (Amersham Biosciences), followed by exposure to chemiluminescence films. Signals were recorded using densitometry.

To analyze ERK1/2 and AKT1 activation, HUVECs after 8 h of serum starvation were treated with bovine FGF2 (FGF2; R & D Systems) or human VEGFA165 (VEGFA; PeproTech) at 10 ng/ml for 0–180 min. Additional cells were treated with FGF2 or with VEGFA for 10 min in the absence or presence of PD98059 (a MEK1/2 inhibitor; 10 μM; 1-h pretreatment) or LY294002 (a PI3K inhibitor; 1.25 μM; 1-h pretreatment). Dimethyl sulfoxide was used as the vehicle control. Phospho-ERK1/2 and -AKT1 and total ERK1/2 and AKT1 were analyzed. Both kinase inhibitors were purchased from Calbiochem.

To determine whether PCN enhanced activation of FGF receptor 1 (FGFR1) and kinase insert domain receptor (KDR, also termed VEGFR2; a type III receptor tyrosine kinase)—major receptors for FGF2 and VEGFA, respectively—HUVECs were treated without or with FGF2 or VEGFA. Total phosphotyrosine proteins in protein samples (600 μg) were immunoprecipitated (IP) with agarose-conjugated p-Tyr (PY99) antibody (Santa Cruz Biotechnology) as described previously [23]. After washing, the IP sample was subjected to Western blotting for FGFR1 and VEGFR2 using three antibodies against different epitopes of FGFR1 and one antibody against VEGFR2, respectively. Additional cell lysates were subjected to immunoblotting directly with a phospho-FGFR1 antibody.

Cell Proliferation and Migration Assays

Cell proliferation assay was performed using a bromodeoxyuridine (BrdU) kit (EMD) as described previously [24]. Cells seeded in 96-well plates (8000 cells per well) were cultured for 16 h. After 8 h of serum starvation, cells were treated with FGF2 or VEGFA for 16 h, labeled with BrdU for 8 h, fixed, and probed with anti-BrdU antibody. Signals were detected using a microplate reader (Synergy HT Multi-Mode; BioTek).

Cell migration was evaluated using a 24-multiwell FluoroBlok transwell insert system (BD Biosciences) [25]. After 8 h of serum starvation, cells were seeded into inserts, followed by addition of FGF2 or VEGFA in the bottom wells. After 16 h of incubation, calceinacetoxymethyl ester, a fluorescent dye (final concentration 0.5 μg/ml; Invitrogen) was added to the bottom wells and incubated for 30 min. Migrated cells were recorded (four randomly chosen fields per well) under a Nikon inverted microscope connected with a CCD camera. Cell numbers were counted using Metamorph image analysis software (Molecular Devices).

To determine the sustainability of the SCN- or PCN-programmed cell proliferation and migration, SCN and PCN cells were precultured under 3% O_2 and 21% O_2 (note: the O_2 levels were reversed), respectively, followed by the BrdU and migration assays. Briefly, the SCN and PCN cells were cultured under 21% and 3% O_2 until reaching confluence, and were then switched to 3% and 21% O_2, respectively, for 5, 2, 1, or 0 days. For example, cells in the Day 1 group were switched to the different O_2 culture condition 4 days after the cells in the Day 5 group were switched. Thus, all bioassays were conducted on the same day. Throughout the experiment, media were changed every 48 h. All 3% O_2 experiments were conducted in the hypoxic chamber with media preequilibrated to 3% O_2.

To examine the effects of MEK1/2 and PI3K pathways on cell proliferation and migration, additional cells were pretreated with PD98059, U0126 (another MEK1/2 inhibitor; Calbiochem), LY294002, or dimethyl sulfoxide (vehicle) for 1 h, followed by the proliferation and migration assays.

Statistical Analyses for All Studies Other Than Microarray Analysis

Data were analyzed using one-way analysis of variance (SigmaStat; Jandel Co.). When an F test was significant, data were compared with their respective control by Bonferroni multiple comparisons or Student *t*-test.

RESULTS

Characterization of HUVECs

To verify the endothelial phenotype and purity of HUVECs used, multiple approaches have been conducted. We found that both SCN and PCN HUVECs exhibited a cobblestone morphology at confluence, positive uptake of DiI-Ac-LDL (≥98%), and CD31 positivity (≥96%), and they formed capillarylike tube structures (Supplemental Fig. S1), indicating establishment of a highly pure population of primary HUVECs under both SCN and PCN. We further measured the cell size (see Supplemental Methods) and observed that the attached PCN cells exhibited a larger cell area in a spread state, whereas the diameter of cells in suspension was similar between SCN and PCN cells (Supplemental Fig. S1). This observation also indicates morphological adaptations of endothelial cells to different O_2 levels, strongly implying alternations of cell behaviors, possibly caused by differential gene expression.

Microarray Analysis

To investigate the effects of PCN on global gene expression in HUVECs, microarray analysis was performed. Compared with SCN, PCN up-regulated 41 genes and down-regulated 21 genes (Supplemental Table S3). Two-dimensional hierarchical clustering of DE genes and cell lines revealed similar expression signatures within each culture condition but very different expression signatures between SCN and PCN cells (Fig. 1A). The IPA for these DE genes demonstrated a prominent enrichment for genes relevant to cell cycle, cell death, cellular movement, and cellular development (Fig. 1B). The IPA also showed several highly associated networks (Fig. 1C), in which MEK1/MEK2/ERK1/ERK2 and PI3K/AKT1 were located in the hubs of gene interaction networks, suggesting the importance of interactions between these signaling pathways and PCN-programmed gene expression. Thus, PCN induces dramatic changes in the transcriptional profiles in HUVECs, which are highly relevant to many essential cell functions (e.g., cell proliferation and migration) and underlying key signaling pathways.

To verify the microarray results, nine genes (six DE and three unchanged genes) were selected for qPCR analysis (Fig. 2A). We found that except for *HES1*, the other eight genes exhibited changes similar to those in the microarray analysis ($R^2 = 0.88$; Fig. 2B). In addition, 12 proteins were examined by Western blotting (Fig. 2C). A total of 83% of examined proteins (CDCP1, NOS3, HIF2α, FGFR1, VEGFR1, VEGFR2, ERK1/2, AKT1, and β-actin) exhibited changes similar to their mRNA counterparts. Conversely, PCN elevated *BNIP3* mRNA but not protein, whereas it increased HIF1α protein but not mRNA levels (Fig. 2C). This inconsistency between protein and mRNA levels is not surprising because the mRNA-protein expression could be poorly correlated in many genes, possibly because of posttranscriptional regulation [26]. As expected [1, 2, 27], PCN increased HIF1α protein but not mRNA levels in HUVECs. Thus, for most genes the data resulting from the microarray analysis are compatible to those from qPCR and Western blotting.

It is noted that PCN increased the mRNA of *VEGFC*, whereas it did not alter mRNA and/or protein levels of VEGFA, FGF2, FGFR1, VEGFR1, and VEGFR2 (Fig. 2C and Supplemental Table S3). Moreover, 95% probe sets of FGFR1 were present, whereas 89% of probe sets of *FGFR2*, 63% of *FGFR3*, and 100% of *FGFR4* were absent, indicating that *FGFR1* is the major FGF receptor in HUVECs.

Cell Proliferation and Migration

To determine the role of PCN in endothelial function, cell proliferation and migration assays were performed. We observed that as FGF2 and VEGFA promoted SCN and PCN cell proliferation, PCN further enhanced FGF2- and VEGFA-stimulated cell proliferation (Fig. 3A). Preculturing SCN cells in 3% O_2 time-dependently promoted cell proliferation; however, preculturing PCN cells in 21% O_2 attenuated cell proliferation (Fig. 3C). More importantly, even up to 5 days,

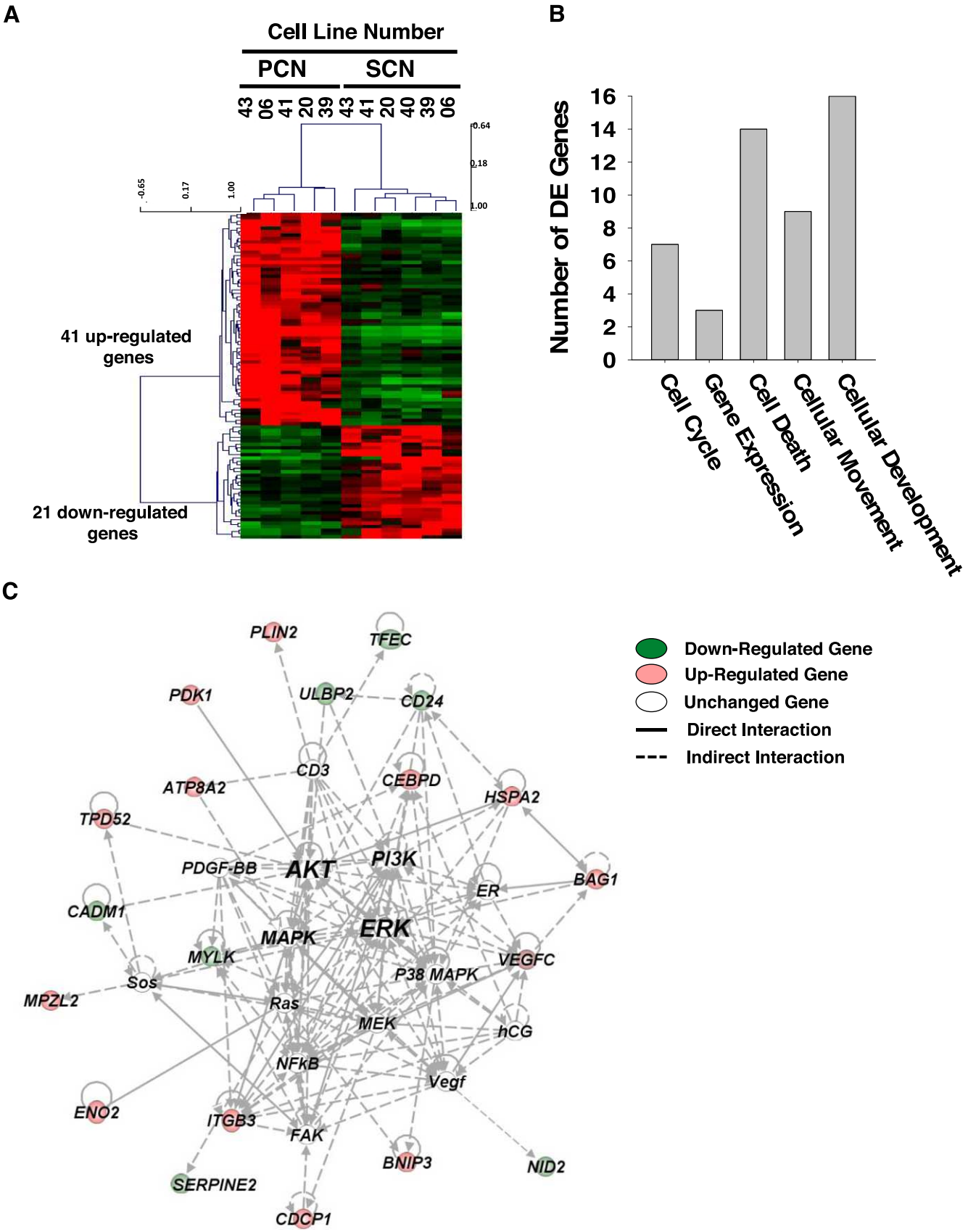
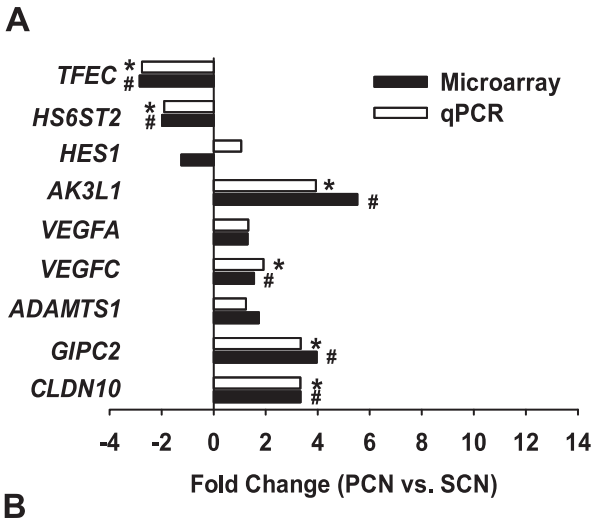
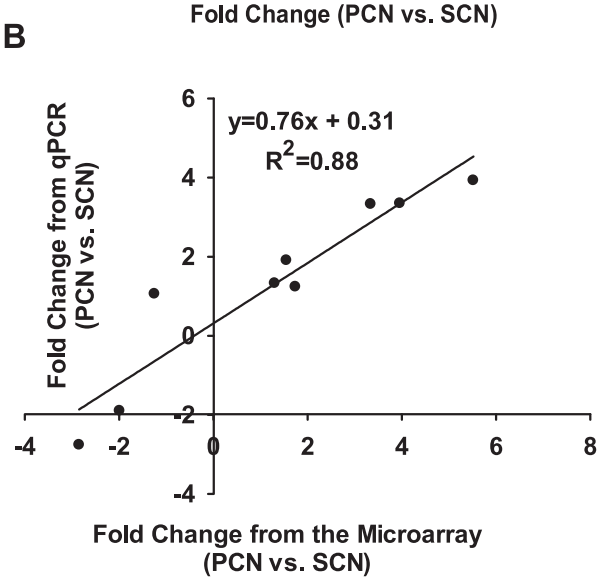
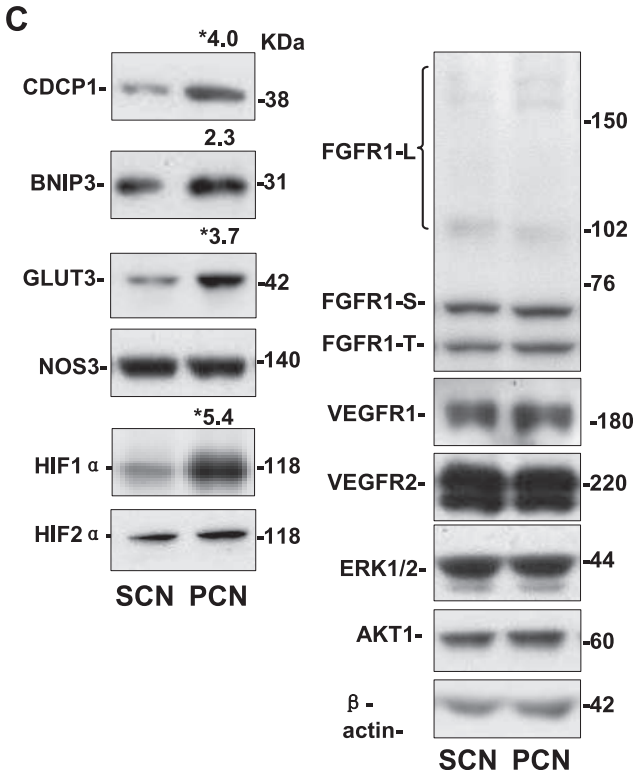


FIG. 1. Microarray analysis and IPA. **A**) Unsupervised two-dimensional hierarchical clustering (Pearson correlation) of the probe sets. **B**) The IPA for cellular functions of PCN-induced DE (as relative to SCN) genes. **C**) The IPA of PCN-regulated gene networks. *ATP8A2*, ATPase, aminophospholipid transporter, class I, type 8A, member 2; *BAG1*, BCL2-associated athanogene; *BNIP3*, BCL2/adenovirus E1B 19-kDa interacting protein 3; *CADM1*, cell adhesion molecule 1; *CD24*, CD24 molecule; *CDCP1*, CUB domain-containing protein 1; *CEBPD*, CCAAT/enhancer-binding protein, delta; *ENO2*, enolase 2; *HSPA2*, heat shock 70-kDa protein 2; *ITGB3*, integrin, beta 3; *MPZL2*, myelin protein zero-like 2; *MYLK*, myosin light chain kinase; *NID2*, nidogen 2; *PDK1*, pyruvate dehydrogenase kinase, isozyme 1; *PLIN2*, perilipin 2; *SERPINE2*, serpin peptidase inhibitor, clade E, member 2; *TFEC*, transcription factor EC; *TPD52*, tumor protein D52; *ULBP2*, UL16-binding protein 2; *VEGFC*, vascular endothelial growth factor C.







such O_2 changes did not completely eliminate PCN- and SCN-programmed cell proliferative responses to FGF2 and VEGFA.

Both FGF2 and VEGFA stimulated SCN and PCN cell migration (Fig. 3B and Supplemental Fig. S2). Physiological chronic normoxia further promoted such stimulatory effects (Fig. 3B). Because PCN slightly enhanced FGF2-stimulated cell migration, the sustainability of the SCN- or PCN-programmed cell migration was evaluated only for the VEGFA effects. Preculturing SCN cells in 3% O_2 time-dependently promoted VEGFA-stimulated SCN cell migration, whereas preculturing PCN cells in 21% O_2 attenuated VEGFA-stimulated PCN cell migration (Fig. 3D). Such O_2 changes up to 5 days also did not completely abolish PCN- and SCN-programmed cell migratory response to VEGFA.

These results clearly show that PCN indeed enhances cell proliferation and migration in response to FGF2 and VEGFA, and such O_2 programmed effects are relatively stable.

Activation of the MEK1/MEK2/ERK1/ERK2 and PI3K/AKT1 Pathways

We next examined the effects of PCN on activation of the MEK1/MEK2/ERK1/ERK2 and PI3K/AKT1 pathways, two of the critical pathways in regulating cell function, in HUVECs. We found that basal levels of phospho-ERK1/2 (pERK1/2) and phospho-AKT1 (pAKT1) at time 0, and total ERK1/2 (tERK1/2) and total AKT1 (tAKT1) at all time points studied were similar between SCN and PCN cells (Fig. 4). FGF2 and VEGFA rapidly ($\leq$10 min) induced ERK1/2 and AKT1 phosphorylation in both SCN and PCN cells (Fig. 4, A and B). Compared with SCN, PCN further enhanced FGF2- and VEGFA-induced ERK1/2 phosphorylation (Fig. 4A). Physiological chronic normoxia significantly amplified VEGFA-induced AKT1 phosphorylation at 5 min, which temporally shifted the phosphorylation curve of AKT1 to the left (Fig. 4B). In contrast, PCN did not enhance FGF2-induced AKT1 phosphorylation. These data indicate that PCN similarly promotes FGF2- and VEGFA-induced ERK1/2 activation; however, it differentially activates AKT1 in response to FGF2 and VEGFA.

PD98059 (a selective inhibitor of MEK1/2) at 10 μM significantly inhibited FGF2- and VEGFA-induced pERK1/2 without altering pAKT1 in both SCN and PCN cells (Fig. 4C). Similarly, LY294002 (a selective inhibitor of PI3K) at 1.25 μM totally blocked FGF2- and VEGFA-induced pAKT1 without altering pERK1/2 (Fig. 4D), suggesting no cross-talk between these two pathways.

FIG. 2. Quantitative PCR and Western blot analyses. **A** and **B**) Comparison and correlation between qPCR and microarray data. Data are expressed as mean fold of SCN. *,#Different from SCN for each analysis ($P \leq 0.05$; n = 5 or 6 pairs of cell preparations). A total of six DE and three unchanged genes were subjected to qPCR analysis. *TFEC*, transcription factor EC; *HS6ST2*, heparan sulfate 6-*O*-sulfotransferase 2; *HES1*, hairy and enhancer of split 1; *AK3L1*, adenylate kinase 3-like 1; *VEGFC*, vascular endothelial growth factor C; *ADAMTS1*, ADAM metallopeptidase with thrombospondin type 1 motif 1; *GIPC2*, GIPC PDZ domain-containing family, member 2; *CLDN10*, claudin 10. **C**) Western blot analysis for PCN-regulated and unregulated gene products. Data normalized to β-actin are expressed as mean fold of SCN. *Different from SCN ($P \leq 0.05$; n $\geq$ 4 pairs of cell preparations). CDCP1, CUB domain-containing protein 1; BNIP3, BCL2/adenovirus E1B 19-kDa interacting protein 3; NOS3, nitric oxide synthase 3 (also termed eNOS); HIF2α, hypoxia inducible factor 2, alpha subunit; FGFR1-L, FGFR1-S, and FGFR1-T, fibroblast growth factor receptor 1 long, short, and truncated forms, respectively; VEGFR1, VEGF receptor 1.

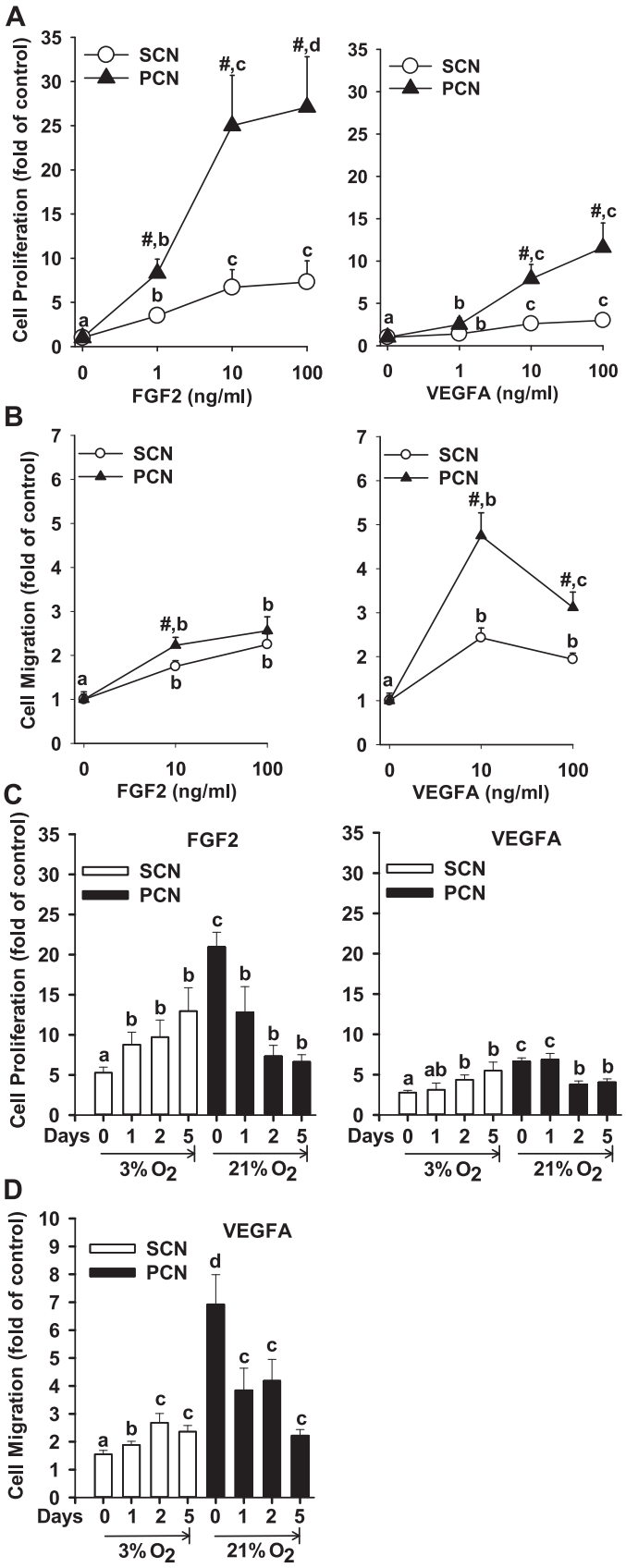


FIG. 3. Physiological chronic normoxia enhances FGF2- and VEGFA-stimulated proliferation and migration of HUVECs. **A** and **B**) SCN and PCN cells were cultured under 21% and 3% O_2, respectively. **C** and **D**) SCN and PCN cells were preincubated in 3% and 21% O_2, respectively. After serum starvation, cells were treated with FGF2 and VEGFA, followed by the proliferation and migration assays. Data are expressed as means ± SEM fold of the no-growth factor control. #Different from SCN at each corresponding dose ($P \leq 0.05$). a,b,c,dMeans with different letters differ ($P \leq 0.05$); n = 6 pairs of cell preparations.

Effects of PD98059/U0126 and LY294002 on Cell Proliferation and Migration

To further investigate the role of the MEK1/MEK2/ERK1/ERK2 and PI3K/AKT1 pathways in cell proliferation and migration, pharmacological kinase inhibitors were used in cell proliferation and migration assays. The optimal concentrations of these kinase inhibitors were first determined. We observed that in the absence of FGF2 and VEGFA, PD98059 up to 10 μM, U0126 (another selective inhibitor of MEK1/2) up to 1 μM, and LY294002 up to 1.25 μM did not change the basal cell proliferation (Fig. 5), whereas PD98059 at 20 μM, U0126 at 2 μM, and LY294002 at 2.5 μM inhibited it. These data indicate that PD98059 up to 10 μM, U0126 up to 1 μM, and LY294002 up to 1.25 μM alone do not have significant cytotoxic effects on HUVECs.

We also found that PD98059 up to 10 μM and U0126 up to 1 μM dose-dependently inhibited the FGF2- and VEGFA-stimulated PCN but not SCN cell proliferation. More importantly, PD98059 at 10 μM and U0126 at 0.25–1 μM decreased FGF2- and VEGFA-stimulated PCN cell proliferation to levels similar to SCN cell proliferation in the absence of either kinase inhibitor (Fig. 5A), indicating that PD98059 and U0126 completely abolished PCN-enhanced cell proliferation in response to FGF2 and VEGFA. LY294002 at 1.25 μM partially suppressed the PCN and SCN cell proliferative response to FGF2 and VEGFA (Fig. 5B). LY294002 at 1.25 μM also abolished PCN-enhanced cell proliferation in response to FGF2 and VEGFA. For cell migration, neither PD98059 at 10 μM nor LY294002 at 1.25 μM significantly changed basal cell migration in the absence of FGF2 and VEGFA. Both PD98059 and LY294002 suppressed FGF2- and VEGFA-stimulated SCN and PCN cell migration. PD98059 and LY294002 abolished the PCN-enhanced cell migratory response to FGF2, whereas they partially attenuated the PCN-enhanced cell migration in response to VEGFA (Fig. 5C). These data suggest that both MEK1/2 and PI3K are key kinases mediating the PCN-enhanced proliferation and migration of HUVECs stimulated by FGF2 and VEGFA.

Phosphorylation of FGFR1 and VEGFR2 in HUVECs

Because biological actions of FGF2 and VEGFA are largely dependent on activation of their receptor tyrosine kinases, we examined whether PCN enhanced phosphorylation (as an indicator of the receptor activation) of FGFR1 and VEGFR2 in response to FGF2 and VEGFA. We saw that the basal phosphorylation levels of FGFR1 and VEGFR2 were similar between SCN and PCN cells. In the immunoprecipitation assay, all three different FGFR1 antibodies used were unable to detect FGF2-increased phosphorylation of overall FGFR1 isoforms (Supplemental Fig. S3) and each individual isoform (data not shown). However, directly probing FGFR1 phosphorylation at Tyr653/654 revealed that FGF2 increased FGFR1-L phosphorylation in both SCN and PCN cells (Fig. 6A). Physiological chronic normoxia slightly enhanced such phosphorylation, but this enhancing effect did not reach statistical significance. In contrast, VEGFA significantly increased VEGFR2 phosphorylation in SCN and PCN cells,

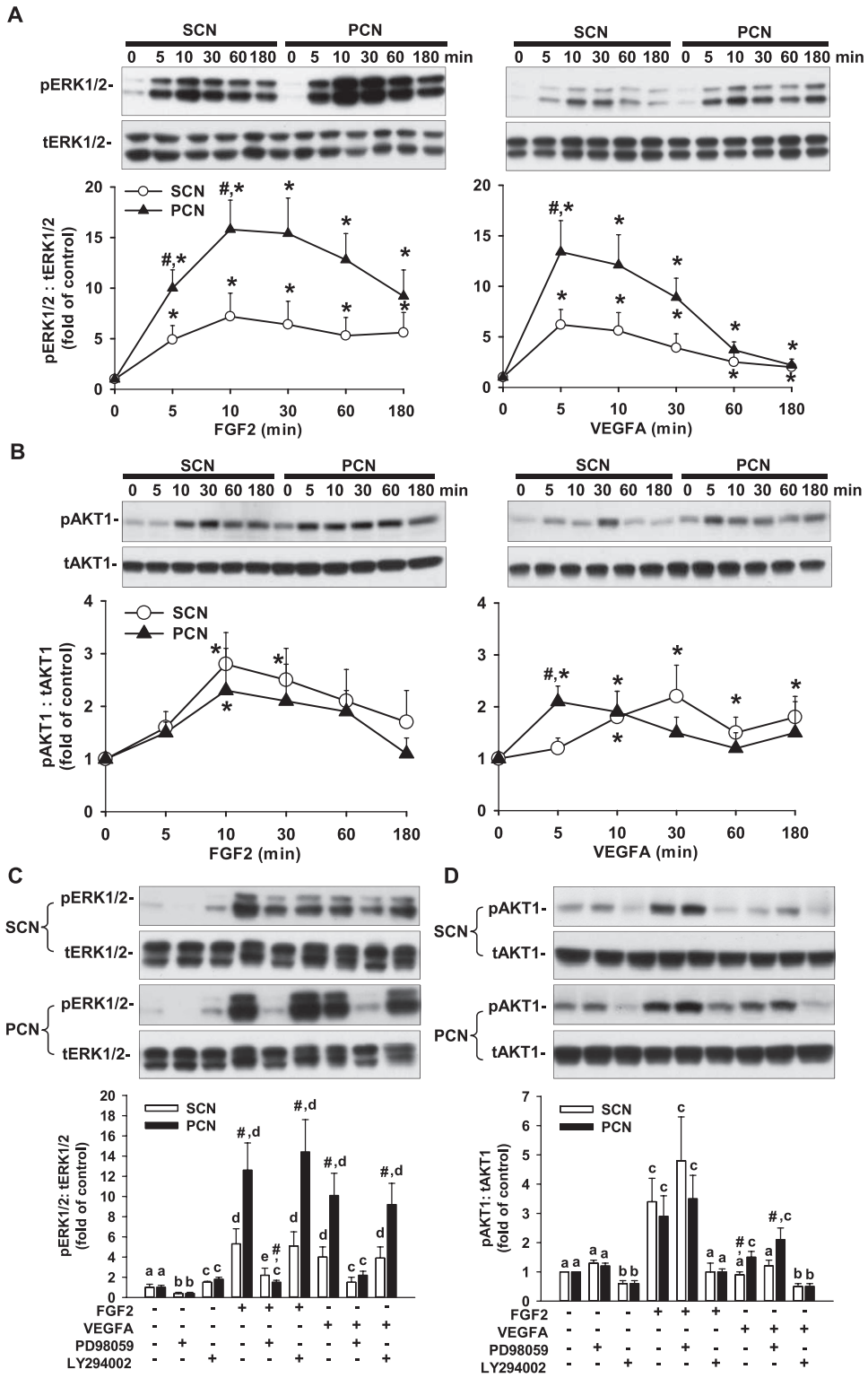


FIG. 4. Effects of PCN on FGF2- and VEGFA-induced ERK1/2 and AKT1 phosphorylation. **A** and **B**) After serum starvation, cells were treated with 10 ng/ml FGF2 or VEGFA. **C** and **D**) Cells were treated with FGF2 (10 min) or VEGFA (10 min) in the absence or presence of PD98059 (10 μM) or LY294002 (1.25 μM). Data normalized to tERK1/2 or tAKT1 are expressed as means ± SEM fold of the time 0 control. *Different ($P \leq 0.05$) from the time 0 control. #Different ($P \leq 0.05$) from SCN at each corresponding time point. [a,b,c,d]Means with different letters differ in SCN or PCN cells ($P \leq 0.05$); n = 6 pairs of cell preparations.

and PCN further promoted such phosphorylation (Fig. 6B). Thus, PCN differentially mediates activation of FGFR1 and VEGFR2 in response to FGF2 and VEGFA, respectively, in HUVECs.

DISCUSSION

In this study, we have established HUVEC models in vitro within the range of physiological chronic O_2 levels [1–4] and also demonstrated that PCN induces dramatic changes in the

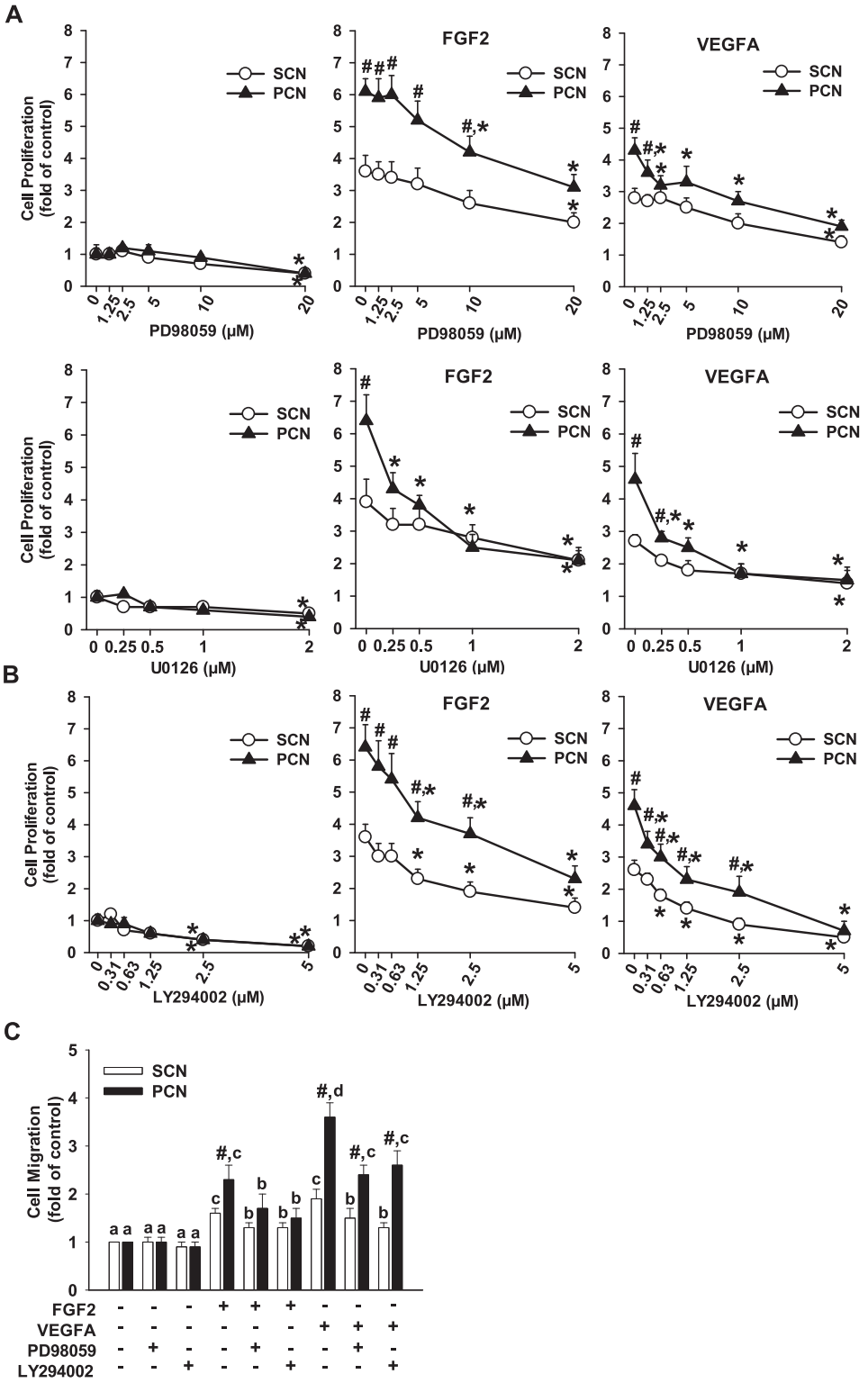


FIG. 5. Effects of PD98059/U0126 and LY294002 on FGF2- and VEGFA-stimulated SCN and PCN proliferation and migration of HUVECs. **A** and **B**) After serum starvation, cells were treated with 10 ng/ml FGF2 and VEGFA in the absence or presence of PD98059/U0126 and LY294002, followed by the proliferation assay. **C**) After serum starvation, cells were treated with 10 ng/ml FGF2 and VEGFA in the absence or presence of PD98059 (10 μM) and LY294002 (1.25 μM), followed by the migration assay. Data are expressed as means ± SEM fold of the no-growth factor control. #Different from SCN at each corresponding dose ($P \leq 0.05$; n = 10 pairs of cell preparations). *Different from the no-inhibitor control ($P \leq 0.05$; n = 10 pairs of cell preparations). [a,b,c,d]Means with different letters differ in SCN or PCN cells ($P \leq 0.05$; n = 6 pairs of cell preparations).

transcriptional profiles in human endothelial cells. In parallel, PCN significantly enhances FGF2- and VEGFA-stimulated cell proliferation and migration, and ERK1/2 activation. These PCN-enhanced cell responses are associated with increases in phosphorylation, but not expression of major FGF and VEGFA receptors. Moreover, these PCN-enhanced cell proliferations and migrations are mediated via the MEK/ERK1/ERK2 and PI3K/AKT1 pathways. Interestingly, these PCN-programmed

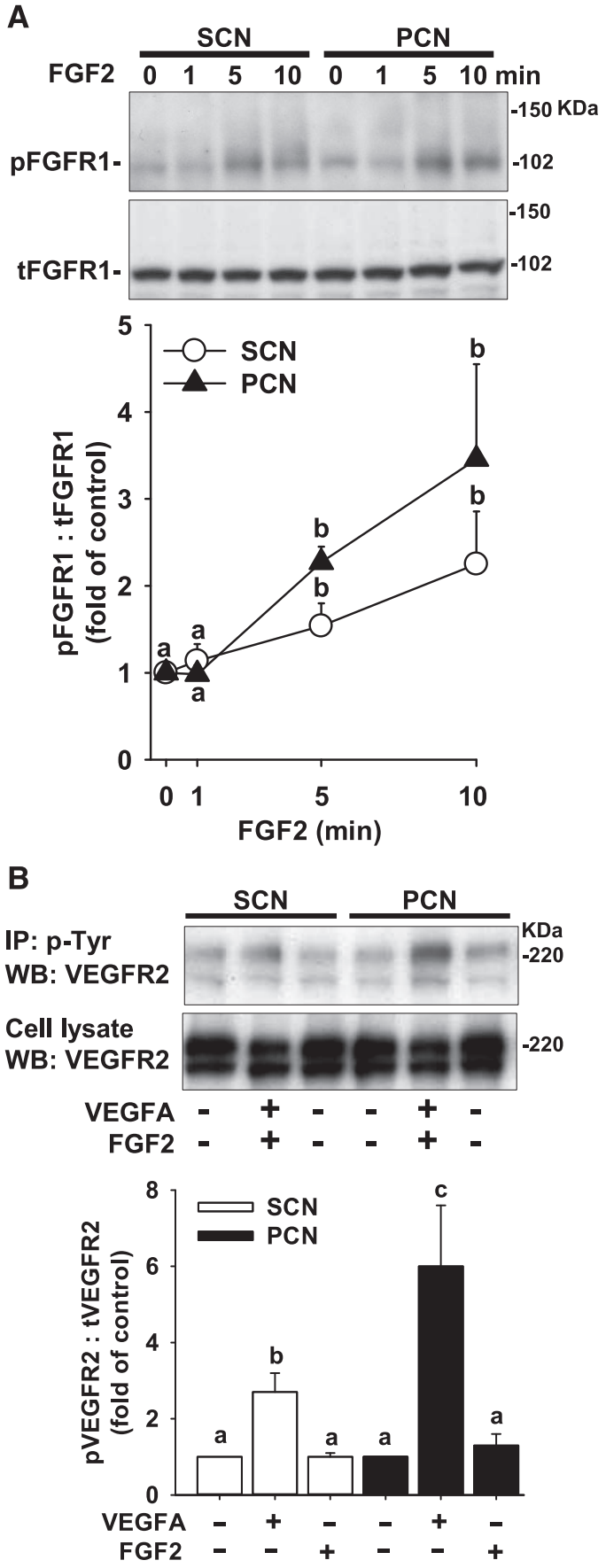


FIG. 6. Physiological chronic normoxia enhances VEGFA-induced VEGFR2 but not FGF2-induced FGFR1 phosphorylation in HUVECs. **A**) After serum starvation, cells were treated with FGF2 (10 ng/ml). Proteins were subjected to Western blotting for phospho-FGFR1 (pFGFR1; Tyr653/654) and total FGFR1 (tFGFR1). **B**) Cells were treated with VEGFA or FGF2 (10 ng/ml; 10 min), serving as a negative control. Proteins were IP with agarose-conjugated p-Tyr antibody. The IP samples were subjected to Western blotting for phospho-VEGFR2 (pVEGFR2). Proteins without immunoprecipitation were subjected to Western blotting for total VEGFR2 (tVEGFR2; a loading control). Data normalized to tFGFR1 or tVEGFR2 are expressed as means ± SEM fold of the no-growth factor control. [a,b,c]Means with different letters differ ($P \leq 0.05$; n = 5 pairs of cell preparations).

cell proliferations and migrations are relatively sustainable. Consistent with the fact that endothelial cells physiologically reside under PCN in vivo, these PCN cells represent unique human endothelial models, more closely mimicking the in vivo states.

Given that ambient O_2 actually is extremely hyperoxic to most cells, the cell models established under SCN may represent a subpopulation of cells that adapt to hyperoxia, and their changes in gene profiles induced by acute low-O_2 treatment might not closely reflect the in vivo condition. Indeed, PCN-induced changes in gene profiles as observed in the current study dramatically differ from those induced by acute low-oxygen treatment [28]. For instance, among 62 DE genes induced by PCN in HUVECs, only 6 genes (*AK3L1*, *ENO2*, *PDK1*, *VEGFC*, *CD24*, and *BNIP3*) are identical to those induced by 2% O_2 (up to 48 h) in HUVECs cultured under SCN as reported previously [28]. In particular, in contrast to the report by Scheurer et al. [28], PCN does not increase mRNA and/or protein expression of FGF2, VEGFA, and FGFR1, all of which have been considered as hallmarks of low-O_2 up-regulated genes in endothelial cells [1]. Our data, however, are in agreement with the in vivo evidence that systemic 6% O_2 does not increase *VEGFA* mRNA expression in the endothelial cells of mice [29]. Although caution should be taken to compare our microarray data with those previous reports because different cell preparations and RNA sample preparations might significantly alter gene expression profiles, the current study strongly suggests that PCN-induced transcriptional profiles in endothelial cells, and possibly in many other cells as well, differ radically from the acute low-O_2 induced genes. Strong segregation of SCN- and PCN-regulated genes in unsupervised hierarchical clustering indicates that PCN HUVECs represent a unique cell model to study endothelial cell functions.

In association with the dramatic alternations in transcription profiles, PCN robustly enhances cell proliferation and migration in response to FGF2 and VEGFA, consistent with the effects of acute low O_2 on endothelial cells [11, 12]. It is noteworthy that these PCN- and SCN-programmed cell responses are rather stable, as evidenced by the fact that even up to 5 days of changing O_2 levels cannot completely reverse this chronic O_2 programming (Fig. 3). It is also interesting that the course to reverse the chronic O_2 programmed proliferative responses is much more gradual than those migratory responses. For instance, it took only 1 day of priming PCN cells with 21% O_2 to significantly inhibit PCN-enhanced, VEGFA-stimulated cell migration, whereas more than 2 days of such priming is required to attenuate PCN-enhanced, VEGFA-stimulated cell proliferation. These observations imply that migration of HUVECs is much more sensitive to O_2 changes than proliferation. Further studies are needed to dissect the mechanism underlying such differences.

A follow-up question that needs to be addressed is whether these PCN-enhanced cell responses are mediated via increasing expression of growth factors and their receptors in these cells studied as it could occur in acute O_2-stimulated angiogenesis [1]. In the current study, we do observe that PCN slightly promoted *VEGFC* mRNA expression; however, PCN did not significantly increase mRNA and/or protein expression for FGF2, VEGFA, FGFR1, VEGFR1, and VEGFR2 in HUVECs. In addition, the mRNAs of *FGFR2*, *FGFR3*, and *FGFR4*, as well as FGFR2 protein, were undetectable by microarray analysis or Western blotting. Thus, elevated expression of FGF2 and VEGFA, as well as their major receptors in these cells, is unlikely to play a major role in the PCN-enhanced responses of HUVECs to FGF2 and VEGFA, and other mechanisms must participate in these PCN-induced enhancements. One such mechanism could be via enlarging activation of these receptors upon stimulation by their ligands under PCN. Indeed, PCN enhances VEGFR2 activation, possibly FGFR1 too to some extent, further suggesting that at the receptor levels, PCN-enhanced endothelial functions may be mediated predominantly by increasing sensitivities of growth factor receptors. In this regard, the current microarray data suggest that increasing expression of the adaptors of these receptors may partially contribute to such PCN-increased sensitivities. For example, PCN significantly up-regulates mRNA expression of *GIPC2* (Supplemental Table S3), an adaptor protein of VEGFA receptors that can enhance endothelial proliferation and migration [30].

To date, signaling networks mediating FGF2- and VEGFA-induced human endothelial functions are primarily established on the cell models established under atmospheric O_2. How PCN regulates endothelial functions remains unclear. The IPA suggested that PCN-regulated genes directly or indirectly modulate the function of signaling molecules (Fig. 1). In this study, we focused on the MEK1/MEK2/ERK1/ERK2 and PI3K/AKT1, because both pathways are critical for FGF2- and VEGFA-stimulated endothelial function [8–14]. We demonstrated for the first time that PCN further enhanced FGF2- and VEGFA-induced ERK1/2 activation, and VEGFA-induced AKT1 activation in HUVECs, implying the importance of ERK1/2 and AKT1 in PCN regulation of endothelial functions. The inhibition of PD98059/U0126 and LY294002 on PCN-enhanced, FGF2- and VEGFA-stimulated cell proliferation, as well as VEGFA-stimulated migration, strongly supports this notion. However, intriguingly consistent with our previous report in human placental artery endothelial cells [14], neither PD98059 nor U0126 affected FGF2- and VEGFA-stimulated proliferation of HUVECs under SCN, although PD98059 significantly attenuated FGF2- and VEGFA-induced cell migration and ERK1/2 phosphorylation under SCN. Thus, together with our previous reports [14, 31], it is concluded that both MEK/ERK1/2 and PI3K/AKT1 pathways are critical in FGF2- and VEGFA-stimulated PCN cell proliferation and VEGFA-stimulated migration, whereas only PI3K/AKT1, but not MEK/ERK1/2, is a key pathway in FGF2- and VEGFA-stimulated SCN cell proliferation.

In the current study, PCN transiently enhances VEGFA-induced but not FGF2-induced AKT1 activation; however, PI3K substantially contributes to this PCN-enhanced, FGF2- and VEGFA-stimulated cell proliferation and migration. These data prompted us to propose that although PI3K substantially contributes to these PCN-enhanced, FGF2- and VEGFA-stimulated cell responses, other signals rather than AKT1 may serve as a major signal downstream of PI3K induced by FGF2 under PCN. These potential signals could include pyruvate dehydrogenase kinase isozyme 1 (PDK1) and small GTP-binding protein Rac1, because both of these can also be activated by PI3K [32, 33], and PCN increases PDK1, whereas it decreases CHN2 (Supplemental Table S3), a GTPase-activating protein that can inhibit Rac activity [34].

In conclusion, the current study has clearly demonstrated that PCN programs HUVECs to undergo remarkable changes in global gene expression and other critical cell responses (proliferation, migration, and signaling transduction) to FGF2 and VEGFA. These may prompt us to consider whether we should reexamine most, if not all, knowledge on cellular and molecular mechanisms regulating endothelial growth and function, as suggested in other types of cells [35–38], using cell models established under PCN.

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