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Nitric oxide promotes cell-matrix adhesion of endothelial progenitor cells under hypoxia condition via ITGA5 CpG promoter demethylation

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ABSTRACT

Hypoxia or low oxygen tension causes changes in the structure and functional phenotype of the endothelial progenitor cells (EPCs). EPCs are found to be involved in angiogenesis and vascular repair. However, EPC's role in cell-matrix adhesion under hypoxia conditions is not clearly established. Nitric oxide (NO) exerts a wide range of biological functions, especially in regulating the mobilization and vascular repair of EPCs. In contrast, the link between NO and its role in cell-matrix deadhesion under hypoxia is not studied yet. Here, we investigated the protective role of NO in hypoxia-induced cell-matrix deadhesion of EPCs through an epigenetic mechanism. The EPCs were exposed to 2% hypoxia in the presence or absence of 10 μM Spermine NONOate (NO donor). The result demonstrates that hypoxia exposure intensified mitochondrial oxidative damage and energy defects. Using miScript miRNA qPCR array-based screening, the study found miR-148 as a novel target of hypoxia-induced DNMT1 activation. Mechanistically, the study discovered that hypoxia suppressed miR-148 levels and stimulated EPCs cell-matrix deadhesion via increasing DNMT1 mediated Integrin alpha-5 (ITGA5) CpG promoter hypermethylation. Treatment with a mitochondria-targeted antioxidant, MitoTEMPO, or epigenetic DNMT inhibitor, 5′-azacitidine, or miR-148 overexpression in hypoxic EPCs culture, prevented the cell-matrix deadhesion compared to hypoxic EPCs. Further, treatment of spNO or transient expression of eNOS-GFP attenuated hypoxia-induced cell-matrix deadhesion via inhibition of ITGA5 CpG island promoter methylation. In conclusion, the study provides evidence that NO is essential for cell-matrix adhesion of EPCs by epigenetically mitigating ITGA5 CpG promoter hypermethylation under hypoxia conditions. This finding uncovers the previously undefined mechanism of NO-mediated diminution of hypoxia-induced cell-matrix deadhesion and dysfunction induced by low oxygen tension.

1. Introduction

Endothelial progenitor cells (EPCs) are a special type of cells that differentiated to mature endothelial cells *in vitro* and in vivo and are found to be involved in angiogenesis [1]. The increasing evidence suggested that *ex vivo* expanded circulating endothelial progenitor cells (EPC) play an important role in postnatal neovascularization by restoring the function of damaged organs [2,3]. However, its level was disrupted by different pathophysiological settings such as diabetes, hypercholesterolemia, hypertension, and coronary heart disease [3–6]. These EPCs have been shown to home preferentially to areas of ischemia and to increase neovascularization [7]. A recent study demonstrated that hypoxia is a critical factor that causes endothelial damage and vascular dysfunction through an oxidative stress mechanism [8,9]. The study also reported that the number and migratory activity of EPCs were corroboratively attenuated in coronary artery disease patients and hypoxia [1,10]. Others have shown that homing and neovascularization capacity of EPCs was mediated by beta2-integrins dependent mechanism in a murine model of hind limb ischemia (11). EPCs adhesion to the matrix in the vascular bed is indispensable for neovascularization at the damaged site [11]. However, the precise mechanism by which low oxygen hypoxia-mediated diminution of cell-matrix adhesion of EPCs is yet to be studied.

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Nitric oxide (NO) is an important biologically active vasodilator that is synthesized by NO synthase (eNOS) in vascular endothelium [12]. Decreased NO bioavailability has been associated with vascular damage [12]. The emerging evidence suggested that NO can stimulate EPCs proliferation and mobilization from bone marrow niches to the peripheral circulation to participate in the neovascularization process [12,13]. Others have shown that prolonged exposure of early and late EPCs to hyperglycemic conditions reduces NO bioavailability, and phosphorylation status of eNOS and also their number and proliferative ability [14]. Additionally, Thum et al., 2007 suggested that exposure to hyperglycemic shock to EPCs results in increased NAD(P)H oxidase activity-dependent oxidative damage and reduced NO bioavailability [15]. Sorrentino and colleagues also demonstrated that reversing the oxidative damage by peroxisome proliferator-activated receptor-gamma (PPAR-γ) agonist rosiglitazone restores the NO bioavailability and the in vivo reendothelialization capacity of EPCs in diabetic patients [16]. NO has been reported to be involved in mitochondrial function by binding to cytochrome *c* oxidase of the electron transport system [17]. Nisholi et al. showed that HeLA cells expressing eNOS show an increase in active mitochondrial activity and cytochrome *c* as well as increased mRNA expression of mito-DNA replication machinery [18]. They have also shown that NO produced by eNOS activates a mitochondrial function in HeLA cells through an unknown mechanism.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that consists of 18–25 nucleotides in length. It regulates post-transcriptional mRNA inhibition or degradation in plants and animals [19]. Each miRNA may target multiple genes by pairing with the 3′-untranslated region (3′-UTR) of mRNA. On the other hand, several miRNAs can regulate the same mRNA gene [19]. Previous studies have suggested roles for miRNAs in cell proliferation and migration ability of EPCs [20]. Others have shown that miRNAs are involved in process of angiogenesis by regulating blood vessel formation and repair [21,22]. However, the crosstalk of miRNA regulation and NO function in hypoxia-induced deadhesion of EPCs to matrix has remained unclear.

Therefore, the present study investigated the role of exogenous NO supplementation reverses cell-matrix deadhesion in cultured EPCs under hypoxia. Mechanistically, NO administration restores mitochondrial biogenesis-dependent miR-148 upregulation and recovers cell-matrix adhesion via the DNMT1-ITGA5 axis.

2. Material and methods

2.1. Reagents and chemicals

Spermine NONOate was purchased from Calbiochem (EMD Chemicals Inc., Germany). DAF-FM (4-amino-5-methylamino-2′, 7′-difluorofluorescein), L-NAME (N_ω-Nitro-L-arginine methyl ester hydrochloride), Carboxy-PTIO potassium salt (CPTIO), MitoTEMPO (2-(2,2,6,6-Tetramethylpiperidin-1-oxyl-4-ylamino)-2-oxoethyl) triphenylphosphonium chloride), and was purchased from Sigma Chemical Co (St. Louis, MO). The eNOS-GFP plasmid was purchased from Addgene (Plasmid#22444). Endothelial cell growth medium (EGM-2) supplemented with a bullet kit containing fetal bovine serum (FBS), and growth supplements were purchased from Lonza clonetics (Catalog No:3156).

2.2. Isolation of PBMNC and its differentiation to EPCs

Human peripheral blood (PB) samples (100 ml) were obtained from Rotary Central-TTK-VHS Blood Bank (Chennai, India) with proper consent (n = 7, male). The blood sample was collected into a sterile pack containing citrate phosphate dextrose solution as an anticoagulant (Terumo Pencol Limited, India) and was processed immediately for further analysis. Human PBMNCs were isolated from PB as previously described [23]. Briefly, 7 ml of whole peripheral blood was layered on Histopaque in a 15 ml falcon tube and centrifuged for 20 min at 2200 rpm. The isolated PBMNCs were seeded at the density 20,000 cells/ml in culture plates pre-coated with fibronection (8 μg/ml) (Sigma Aldrich) under EGM-2 media supplemented with bullet kit containing fetal bovine serum (FBS) and growth supplements (Lonza, clonetics, EGM-2 SingleQuots, Catalog No: 3156). Then, PBMNCs were allowed to culture under normoxic (37 °C in 20% O2 and 5% CO2; normoxia) or hypoxic (37 °C in 10% O_2 and 5% CO_2; hypoxia) conditions until its differentiation into EPCs.

2.3. Cell-matrix adhesion assay

The cell adhesion assay was performed as previously described [24]. Briefly, EPCs (2 x 10^5) were seeded in 24-well plates coated with human recombinant fibronectin (10 μg/ml) and incubated for 24 h in both hypoxic and normoxic conditions. For inhibition experiments, EPCs were pre-incubated with Anti-Integrin α5 Antibody (Catalogue-CBL497, Sigma) for 1 h at 4 °C. Then, cells were washed with 1X sterile phosphate buffer saline (PBS) to remove the non-adherent cells. The adherent cells were then trypsinized and counted using a hemocytometer. Further, cells were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) solution for 15 min to visualize cell nuclei.

2.4. Estimation of nitrite and NO production

Intracellular NO and nitrite production in cultured EPCs was measured by the colorimetric Griess assay method as previously described protocol [24]. For nitrite measurement, 150 μl of the cell culture supernatant were mixed with the same volume of Griess reagents (58 mM sulphanilamide in 2.5% phosphoric acid plus 12 mM N-(1-naphthyl)ethylenediamine in 2.5% phosphoric acid) were added, and the samples were incubated for 22 min at 37 °C. The absorbance was read at 570 nm. Nitrite levels were calculated using the sodium nitrite standards curve (1–10 μg). Similarly, Cultured EPCs were incubated with DAF-FM (10 μM) dissolved in PBS for 20 min at 37 °C. The excess DAF-FM probes were washed with sterile 1X PBS. The fluorescence images were taken using a fluorescence microscope. The amount of NO was also evaluated by calculating the mean fluorescence intensity.

2.5. Mitochondrial-specific ROS and mitochondrial mass imaging

Mitochondrial-specific ROS production was detected in EPCs using the MitoSOX™ Red reagent, as previously described [25]. Briefly, EPCs (10^4 cells/well) were grown on a coverslip in the 24-well plates. Following confluency, cells were treated with hypoxia in the absence/presence of spNO (10 μM) and incubated for 24 h. Then, cells were loaded with MitoSOX™ Red reagent (Invitrogen, 1 μM), and incubated in the dark for 15–20 min at 37 °C. The excess probes were washed with 1X PBS and were imaged by confocal microscopy. For imaging of mitochondrial mass, cultured EPCs were incubated with MitoTracker Green FM (Molecular Probes, 500 nM) solution at 37 °C for 22 min and imaged using confocal imaging. For quantitative analyses, fluorescence intensities were assessed in each image using Image J software (NIH) and presented in % of relative fluorescent intensity.

2.6. Lipid peroxidation assay

Lipid peroxidation (malondialdehyde, MDA) levels in EPCs

lysates were estimated using a lipid peroxidation (MDA) assay kit (Colorimetric, ab118970), according to the manufacturer's instructions.

2.7. Glutathione peroxidase (Gpx) activity

The glutathione peroxidases (Gpx) activity in cultured EPCs was measured using a glutathione peroxidase assay kit (Sigma-Aldrich, 353919) as per the manufacturer's instructions.

2.8. Actin cytoskeleton imaging

The imaging of actin filaments was performed using Palladian-Rhodamine staining in the EPCs culture. The cultured EPCs were exposed to hypoxia and normoxia in the presence/absence of spNO treatment. At the end of the treatment procedure, EPCs were fixed under 2% paraformaldehyde and incubated with Invitrogen™ Rhodamine Phalloidin (8 μM, Catalog No.: R415) at 37 °C for 22 min. Fluorescent images of actin filaments in the EPCs were observed under fluorescence microscopy.

2.9. Western blot analysis

The cultured EPCs lysates were prepared using RIPA lysis buffer. The protein lysates were run on 8–10% sodium dodecyl sulfate (SDS)-electrophoresis gel. After blot transfer of proteins, the membrane was blocked with 5% non-fat dry milk (NFDM) solution and probed for primary antibodies (Integrin α5, DNMT1) (1:500). Following wash with 1X TBS-T solutions, the corresponding secondary antibodies conjugated to (HRP) (1:2000) was added. Then, membranes were developed with 1X TMB-H2O2 reagents (Bangalore Genei) as substrate. Band intensities that appeared on the membranes were quantified using Image Lab software.

2.10. microRNA profiling by RT2 miRNA PCR array

Total RNA was isolated in the cultured EPCs using miRNeasy Kit (Qiagen, CA, USA), according to manufacturer's instructions. Total RNA (200 ng as an input) was used to convert into cDNA using the miScript II RT kit (Qiagen). Following cDNA synthesis, the samples were used amplify various miRNA expressions in a 96-well format RT2-miRNA PCR array (MIMM-001Z, miScript miRNA PCR Array Mouse miFinder, Qiagen) using miScript SYBR Green PCR Kit (Qiagen). Further, miRNA-142-5 expression was validated using Mastercycler® ep realplex Real-time PCR System (Eppendorf, Hamburg, Germany). The relative miRNA expression level was calculated by normalization with the U6 expression using the 2-ΔΔCT method. For the mRNA expression analysis, total RNA (1 μg) was used for reverse transcription using Hi-cDNA Synthesis Kit (HiMedia). The mRNA expression of target genes was amplified using MBT108 Hi-SYBr Master Mix (HiMedia). The primers for RT-PCR reactions are purchased from Eurofins and provided the primer sequences in Table 1.

Table 1
Sequences of PCR primers used for real-time quantitative PCR.

Gene	Primer Sequences (5′→3′)
Human DNMT1	FP: AGGTGGAGAGTTATGACGAGGC RP: GGTAGAATGCCTGATGGTCTGC
Human ITGA5	FP: GCCGATTCACATCGCTCTCAAC RP: GTCTTCTCCACAGTCCAGCAAG
Human miR-142-5p	FP: TCAGTTATAAAGGTCAGC RP: GCCTGGGTCACCATGTAG
Human GAPDH	FP: CTCCCACTCTTCCACCTTCG RP: GCCTCTCTTGCTCAGTGTCC

2.11. In silico analysis

Using in silico analysis tool, Targetscan (http://www.targetscan.org/), miRcode (http://www.mircode.org/index.php), we predicted potential targets of miR-142. In addition, using DIANA Tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=lncBase/index), we also confirmed the predicted direct miRNA-142 for DNMT1 expression.

2.12. DNMT1-UTR-3′ luciferase assay

Luciferase activity was performed using DNMT1 miRNA 3′UTR clone (luciferase-RFP reporter). Briefly, EPCs were transfected with DNMT1 miRNA 3′UTR clone (Cat. No-MiUTR3H-00681) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) under a serum-free medium. Following 72 h of post-transfection, the luciferase activity was performed in the cultured lysates using the Dual-Luciferase Reporter Assay (Promega, Madison, WI, USA).

2.13. Overexpression of target miRNA in vitro

To investigate the role of miR-142 function in cultured EPCs under hypoxia exposure, miR-142 mimic (sequence, 5′- UGUAGUGUUUCCUACUUUAUGGA -3'), was purchased (Sigma, USA) and transfected (100 ng/ml) under serum free conditions using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) [19]. After 48 h post-transfection, biochemical, and molecular analyses were performed.

2.14. DNA bioinformatics search and ITGA5 methylation analysis by qMSP

The potential CpG islands in the genomic sequences of the ITGA5 gene, was performed as the protocol previously described [3]. We also used the CpG Island Explorer software (cpgie.sourceforge.net) to discover the nucleotide sequences surrounding the transcription start site (TSS) of ITGA5 and possible enrichment of CpG Islands. Primers, which targeted CpG-rich regions within the ITGA5 region, were generated with MethPrimer Express software (Li-lab, 26). A pair of primers (bisulfite-treated both methylated DNA and unmethylated DNA) was selected for each gene region (Table 1). Briefly, sodium bisulfite conversion of genomic DNA was performed using EZ DNA Methylation-Direct™ Kit (ZYMO RESEARCH, USA). Quantitative methylation-specific PCR (qMSP) was performed to amplify bisulfite converted DNA. The MSP PCR products were amplified using qPCR reactions.

2.15. DNMT activity assay

The total nuclear protein was isolated from the cultured EPCs lysates of various experimental conditions using EpiQuik™ Nuclear Extraction Kit I (Epigentek, Brooklyn, NY, USA). Following protein quantification, DNA Methyltransferase (DNMT) Activity was measured in nuclear protein (12 μg) using EpiQuik™ DNA Methyltransferase Activity/Inhibition Assay Kit (Epigentek) according to the manufacturer's protocol.

2.16. Transfection of eNOS-GFP plasmid by electroporation

To transfect eNOS-GFP pcDNA3 Plasmid in cultured EPCs, the electroporation method was performed as previously described [24]. Briefly, electroporation cuvettes contained 500 μl of EPCs

($1x10^5$) and followed by the addition of 1 μg of eNOS plasmid DNA (in 10 μl). The electric pulses (360 W) were supplied to cuvettes using Electro Cell Manipulator (ECM 600). Then, EPCs were cultured under both normoxic and hypoxic conditions. At the end of the culture, eNOS-GFP expression, NO production, and cell-matrix adhesion was monitored.

2.17. Statistical analysis

Data analyses and graphical presentations were performed with GraphPad Prism, version 9.00 (GraphPad Software, Inc., La Jolla, CA). Data are represented as mean values ± standard error mean (SEM). The experimental groups were compared among the groups by one-way analysis of variance (ANOVA) in combination with Tukey's multiple comparison test. The significance of differences between groups was determined using a Two-tailed, unpaired Student's t-test. $p < 0.05$, $p < 0.01$ and $p < 0.001$ was considered statistically significant.

3. Results

3.1. Nitric oxide (NO) prevents hypoxia-induced mitochondrial oxidative damage and improves mitochondrial biogenesis in endothelial progenitor cells (EPCs)

To demonstrate examine the protecting function of NO donor (spNO) on hypoxia-induced mitochondrial specific oxidative damage and dysfunction, we assessed several parameters of mitochondrial oxidative stress, respiration, and biogenesis in cultured EPCs. Initially, we loaded MitoSOX™ Red, an indicator of mitochondrial superoxide ($O2^{\bullet-}$) production in EPCs. Using confocal imaging, the study showed an increased mitochondrial $O2^{\cdot-}$ generation in the hypoxic (Hyp) EPCs compared to normoxic (Nor) EPCs, but this response significantly prevented NO treatment in Hyp + NO condition (Fig. 1a and b). To prevent mitochondria oxidative damage, we treated mitochondria-targeted specific antioxidant mito-TEMPO (20 μM) in Hyp-EPCs condition and data showed that mito-TEMPO treatment attenuated mitochondrial $O2^{\cdot-}$ generation in the Hyp + mito-TEMPO condition (Fig. 1a and b). On contrary, cellular antioxidant enzyme systems (superoxide dismutase (SOD) activity, glutathione peroxidase (GPx)) was tested. Interestingly, the antioxidant enzymes, SOD, and GPx levels were found to be improved in the Hyp + NO and Hyp + mitoTEMPO conditions compared to the Hyp condition (Fig. 1c and d). We next tested the effect of hypoxia on mitochondrial contents in cultured EPCs by using a MitoTracker™ Green probe imaging. The study showed that mitochondrial mass was significantly increased in the Hyp + NO or Hyp + mito-TEMPO condition compared to the Hyp condition (Fig. 1e). Mitochondrial complex I activity was found to be improved in isolated mitochondria of EPCs from Hyp + NO or Hyp + mito-TEMPO condition compared to Hyp condition (Fig. 1f). Furthermore, mitochondrial ATP production were significantly improved in the isolated mitochondria of Hyp + NO or Hyp + mito-TEMPO condition compared to the Hyp condition (Fig. 1g). Therefore, our study suggests that NO treatments could reverse the hypoxia-linked mitochondrial dysfunction, disruption of mitochondrial biogenesis, and energy defect in cultured EPCs.

3.2. Effect of NO on hypoxia-induced deregulation of miRNAs expression

It has been reported that prolonged hypoxia exposure was associated with microRNA deregulation, however, the effects of hypoxia on microRNA expression need to be studied in cultured EPCs in the context of oxidative stress. Taking this into account, we executed a qPCR-based miRNA array to identify potent miRNAs that would be differentially regulated in hypoxia-conditioned EPCs culture. Our study demonstrated there were many miRNAs were differentially expressed (as represented in hierarchical clustering analysis) under hypoxic simulation in the EPCs compared to normoxia culture (Fig. 2a). Indeed, the study identified the expression of miRNA-142-5p was found to be reduced under hypoxia exposure compared to normoxia condition. Using real-time qPCR assay, the study validated the expression of miRNA-142-5p. The data showed that miRNA-142 was suppressed ($p < 0.05$) in the EPCs culture of Hyp condition, however, miRNA-142 expression was significantly normalized ($p < 0.05$) in the Hyp + NO condition (Fig. 2b). Interestingly, treatment of mito-TEMPO evidently improved the miR-142-5p expression in the Hyp + mito-TEMPO condition (Fig. 2c). These results confirm that hypoxic repression of miR-142-5p expression is mitochondrial-oxidative-dependent in cultured EPCs.

Using *in silico* TargetScan analysis, the study discovered that miR-142 directly targets on the epigenetic regulator, DNA methyltransferase 1 (DNMT1). And also, we found that miR-142 sequence recognized the 263–269 bp of the conserved sequence of the DNMT1-UTR region (Fig. 2d). Further, using qPCR analysis, the study validated that the mRNA transcript level of DNMT1 was significantly increased in the Hyp condition. Indeed, administration of NO or MitoTEMPO and transfection of miR-142 mimic reduced the abnormal increase DNMT1 expression in Hyp + NO or Hyp + mito-TEMPO or Hyp + miR-148 mimic condition compared to Hyp condition (Fig. 2e). In addition, to study further the direct binding interaction of DNMT1 and miR-142, we transfected EPCs with DNMT1 miRNA 3′UTR clone (luciferase-RFP reporter). Our data demonstrated NO, or miR-148 mimic treatment inhibited the luciferase activity of DNMT1 miRNA 3′UTR clone reporter in the Hyp + miR-148 mimic and Hyp + spNO condition compared to the Hyp condition (Fig. 2f). Our data concluded that NO evidently mitigates the epigenetic regulator DNMT1 expression via upregulating the miR-142 expression in hypoxic EPCs culture.

3.3. NO treatment promotes ITGA5 promoter demethylation via the DNMT1-miR-142 axis

Epigenetic DNA methylation is essential for gene silencing and genome stability. This process is initiated by the epigenetic regulator, DNA methyl transferase (DNMTs) which transfers the methyl (-CH3) to the cytosine residue (C-5 position) of the CpG sequence. Fig. 1a depicts the diagrammatic representation of DNMT1 catalyzes DNA methylation at ITGA5 promoter. Therefore, we tested the protein activity of DNMT1 in the cultured EPCs using ELISA (Fig. 3a). The study confirmed that DNMT1 activity was found to be increased under the Hyp condition; however, the above effect was mitigated Hyp + miR-142 mimic and Hyp + spNO condition (Fig. 3a). Likewise, global DNMT activity was significantly increased in the nuclear protein contents of Hyp condition. However, treatment of spNO or miR-142 mimic prevented hypoxia-induced effect (Fig. 3b). As the study found hypoxia was associated with increased epigenetic DNMT1 expression, therefore, we hypothesized that hypoxia would cause promotor hypermethylation of integrin gene (Integrin α5 (ITGA5)). Using ITGA5 promotor methylation analysis via Methprimer (www.urogene.org/methprimer/), the study found that there were 2 CpG islands (Island 1: 182 bp (−48 to − 229) and Island 2: 105 bp (2298–2402) located at the promotor of the ITGA5 gene (Fig. 3d). Our methylation-specific qPCR (qMSP) analysis demonstrated that hypoxia was associated with increased ITGA5 promoter (Island 2 but not Island 1) methylation in cultured EPCs. Interestingly, increased ITGA5 hypermethylation was significantly lessened in the Hyp + miR-142 mimic and Hyp + spNO condition (Fig. 3e). In addition, administration of epigenetic DNMT inhibitor

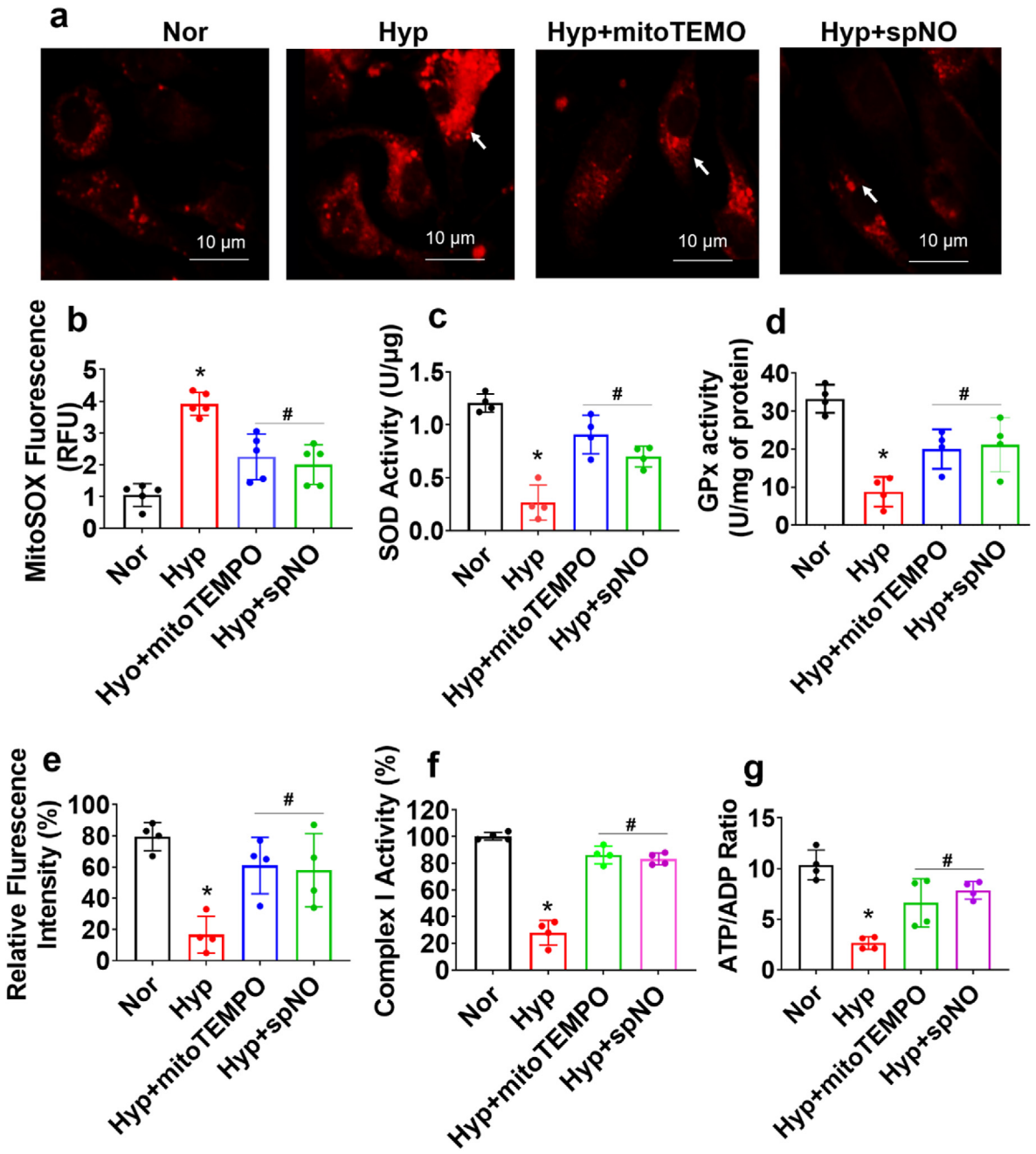

Fig. 1. ***Effect of nitric oxide (NO) on hypoxia-induced mitochondrial damage in cultured EPCs.*** *(a, b) Fluorescence imaging of mitochondrial-specific ROS using MitoSOX red staining (a). Arrow head depicts the mito-ROS staining. The relative fluorescence intensity (RFU) was quantified by ImageJ software (b). Scale bar = 10 μm. (c) SOD activity assay was performed. (d) GPx activity was quantified. (e) Mitochondrial contents were imaged using MitoTracker Green FM staining. Scale bar: 10 μm. (f) Complex I activity was measured. (g) ATP production were measured in isolated mitochondria derived from the EPCs. Experiments were repeated at least three times. Data are expressed as mean ± SEM. n = 4 samples per group. *p<0.05 compared with the control Nor (normoxia), #p<0.05 compared with the Hyp (hypoxia) group. The error bars represent the s.e.m. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)*

(5-Aza, 20 μM) further inhibited the ITGA5 methylation in the Hyp+5-Aza condition compared to the Hyp condition (Fig. 3e). Further, mRNA expression of ITGA5 was improved under the Hyp + miR-148 mimic, Hyp + spNO, and Hyp+5-Aza condition (Fig. 3f). This result concludes that DNMT1-induced ITGA5 promotor methylation contributed to decreased cell adhesion protein, ITGA5 expression under hypoxia exposure via reducing miR-142 expression.

3.4. NO treatment improves integrin α5 expression and cell-matrix adhesion in EPCs

As Integrin α5 (ITGA5) expression is drastically reduced in cultured EPCs under hypoxia insult and treatment of NO donor (spNO) was able to restore it via the miR-142-DNMT1/ITGA5 axis. We further performed a cell-matrix adhesion assay *in vitro*. Briefly, EPCs were seeded on 24-well plates that were pre-coated with

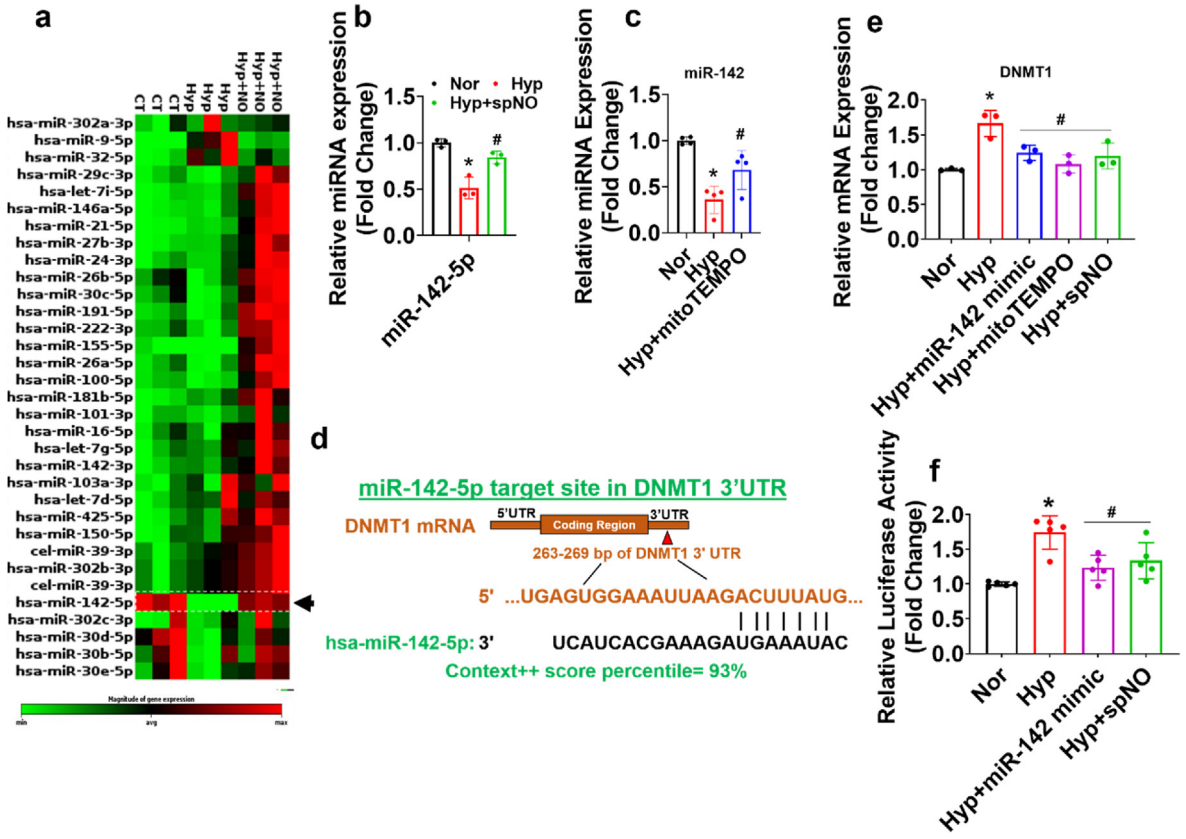

Fig. 2. ***Effect of NO on Hypoxia-induced epigenetic alternation via downregulation of miR-142-5p.*** *(a) miRNA profiling was performed in cultured EPCs (n=3). (b) The expression of miR-142-5p was validated using qPCR assay. (c) Effect of mitoTEMPO on miR-142 expression. (d) Tragetscan analysis of the miR-142 binding site in 263-269 bp of DNMT1 3′-UTR region. (e) mRNA transcript of DNMT1 were assessed. (f) DNMT1 luciferase was performed. Data are expressed as mean ± SEM. n = 3-5 samples per group. *p<0.05 compared with the control Nor (normoxia), #p<0.05 compared with the Hyp (hypoxia) group. The error bars represent the s.e.m.*

fibronectin (10 μg/ml) overnight and allowed to culture under normoxia and hypoxia. The data revealed that spNO or mitoTEMPO or miR-142 mimic or 5-Aza treatments improved cell-matrix adhesion under hypoxia-induced conditions comprared to hyp condition alone (Fig. 4a). In contrast, treatment of NOS inhibitor (L-NAME) and neutralizing integrin α5 antibody treatment in hypoxia culture substantially reduced cell-matrix adhesion in Hyp + L-NAME and Hyp + integrin α5 antibody conditions compared to Hyp condition (Fig. 4a). Additionally, overexpression of eNOS (eNOS-GFP transfection) in EPCs was found to be improved the cell-matrix adhesion in Hyp + eNOS-GFP condition compared to Hyp condition (Fig. 4b).

To further study the mechanical action of hypoxia on the cellular actin network and polymerization, we stained the cultured EPCs with Rhodamine Phalloidin Reagent and imaged F-actin distribution. The data showed a uniform distribution of reorganized F-actin fibers under normoxic conditions (Fig. 4c and d). However, the poorly stained actin stress fibers in the cytoplasm of cells were observed in hypoxic EPCs, instead, cytoplasmic F-actin was preferentially located within fibers at the periphery of the cell. In contrast, this subcellular pattern was restored in both Hyp + spNO conditions (Fig. 4c and d). Collectively, our data demonstrate that NO is indeed indispensably required to restore the cell-matrix adhesion in hypoxic EPCs culture via the miR-148-DNMT1/ITGA5 axis.

4. Discussion

In the current study, we demonstrated that the cell-matrix adhesion property of EPCs was significantly impaired in hypoxic condition, accompanied by increased mitochondrial damage and subsequently alternation of epigenetic DNMT1 activity. Mechanistically, increased mitochondrial damage was associated with the mitochondrial energy defect, downregulation of miR-142-5p expression and leads to activation the epigenetic mechanism of DNMT1 expression. This subsequently down-regulates cell-matrix adhesion protein, integrin α5 expression via hypermethylation of its promoter. The overall molecular and epigenetic changes further accentuate deadhesion of EPCs to the matrix and alternation of actin cytoskeleton network in hypoxic EPCs as compared to normoxia EPCs. Interestingly, the administration of NO donor (spNO) or mitoTEMPO reversed these phenotypes. Our study uncovered the previously undefined role of NO level may play a vital role in attenuating the hypoxia-induced mitochondrial stress and improving the inform distribution pattern of the actin cytoskeleton that indeed revamps the cell-matrix adhesion of EPCs via DNMT1-miR-148/ITGA5 axis.

Hypoxia is a condition in which the body or organs is supplied with inadequate oxygen. It occurs when people rapidly ascend to high altitude or suffer ischemia to particular organs such as myocardial, and cerebral ischemia [24]. In general, hypoxia can promote endothelial cell injury and cause vascular dysfunction and folic acid mitigate the hypoxia [22]. Other have shown that suppression of NADPH oxidase-dependent ROS signaling attenuates hypoxia-induced dysfunctions of endothelial progenitor cells [21]. However, the pathophysiological effect of hypoxia on cell-matrix deadhesion of EPCs is not investigated yet. In the present study, we demonstrated that hypoxia simulation significantly ablated cell-

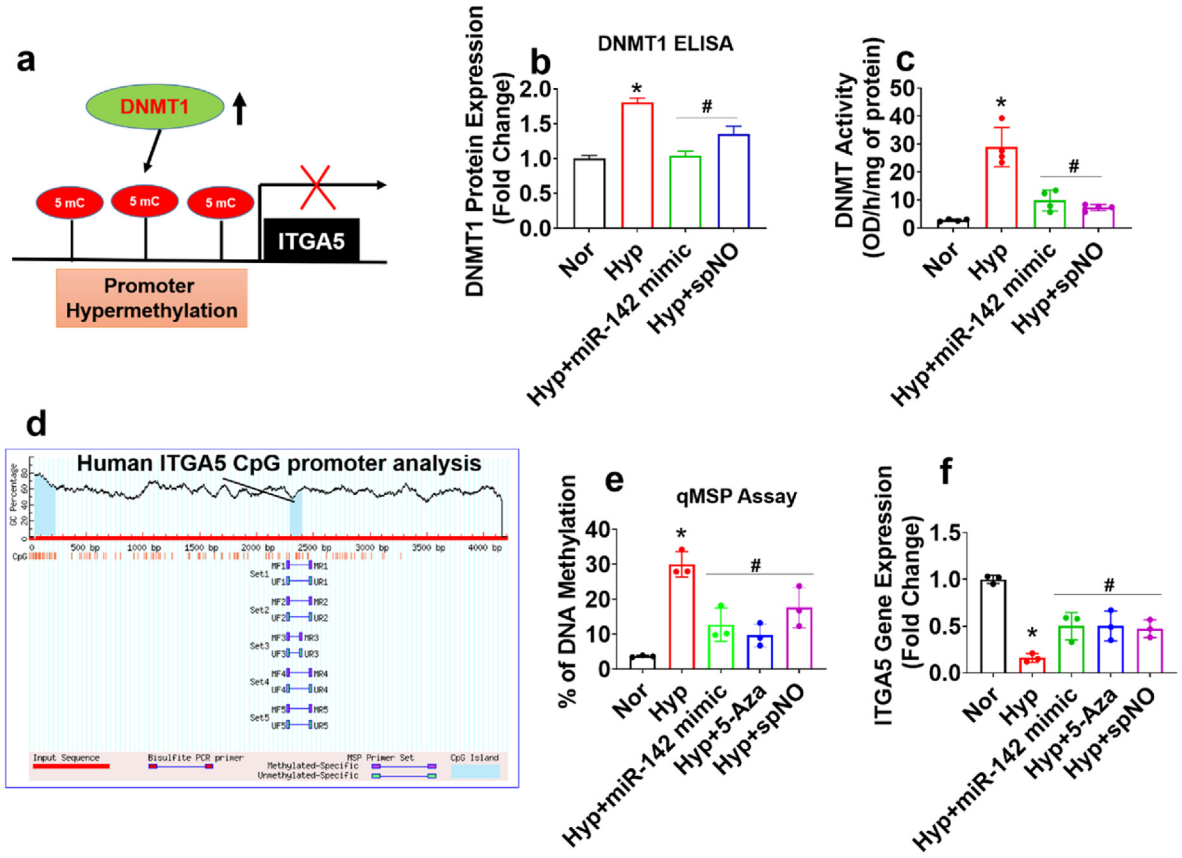

Fig. 3. ***Effect of NO on DNMT1-dependent hypermethylation of ITGA-5 promoter under hypoxia in EPCs.*** *(a) ITGA5 promoter DNA methylation is governed by DNMT1 activity. (b) ELISA assay of DNMT1 protein activity. (c) Global DNMT activity was quantified. (d) Methprimer software analysis of identification of the CpG islands in the ITGA-5 promotor. (e) % of ITGA-5 promoter DNA methylation following qMSP analysis (f) mRNA transcript expression of ITGA-5. Data are expressed as mean ± SEM. n = 3-4 samples per group. *p<0.05 compared with the control Nor (normoxia), #p<0.05 compared with the Hyp (hypoxia) group. The error bars represent the s.e.m.*

matrix adhesion of EPCs via down-regulating integrin α5 (ITGA-5) expression as compared to control (Figs. 3 and 4). However, supplementation of NO mitigates the above effect.

DNA methylation is one of the important epigenetic mechanisms that occur at the CpG island of the target gene and thereby it regulates gene transcription [26]. However, the prospective role of epigenetic DNA methylation in cell-matrix adhesion under hypoxia insult needs to be investigated in EPCs. Using *in silico* Targetscan analysis and qMSP analysis, the study confirmed that a high degree of DNA methylation was detected at the CpG sites of the ITGA-5 promoter. Furthermore, our qPCR data demonstrate that ITGA-5 expression was significantly reduced through increased CpG DNA hypermethylation in the hypoxic EPCs condition compared to the normoxia condition. However, the administration of epigenetic DNMTs inhibitor, 5-Aza, and NO donor mitigated the above effect in the Hyp+5-Aza and Hyp + spNO conditions (Fig. 3). As hypoxia exposure dramatically suppressed miR-142 expression, which indeed upregulated DNMT1. This resulted in reduced ITGA-5 expression (Fig. 3) and loss of cell-matrix adhesion in EPCs culture (Fig. 4). Mitochondrial function and biogenesis are associated with oxidative phosphorylation and energy homeostasis. However, the link between mitochondrial damage and miRNA deregulation in EPCs is not clear under hypoxia insult. The current study found that hypoxia-induced mitochondrial damage was associated with reduced miR-142 expression and subsequent epigenetic changes. Intriguingly, the administration of mitoTEMPO improved mitochondrial function and energy production (Fg. 1). On the other hand, mitoTEMPO treatment significantly increased miR-142 expression in the Hyp + mitoTEMPO condition compared to the Hyp condition (Fig. 2).

The eukaryotic cell express α5β1 integrin, a cell surface receptor that binds to matrix adhesion protein fibronectin [1]. These receptors are implicated in cell migration, proliferation, differentiation, and adhesion processes. However, its mechanistic role in EPC's adhesion to matrix and actin cytoskeletal arrangement under hypoxia conditions is not established. In the present study, we showed that reduced ITGA-5 expression was associated with decreased cell-matrix adhesion in cultured EPCs under hypoxia *in vitro*. However, spNO or overexpression of miR-142 improves integrin α5 expression and cell-matrix adhesion suggesting that this integrin receptor is critical in cell-matrix adhesion (Fig. 4). Additionally, 5-Aza treatment could suppress epigenetic DNMT1 dependent hypermethylation and subsequently improves cell-matrix adhesion (Fig. 4). Furthermore, supplementation of spNO could restore the normal localization of actin filaments and provide a basal morphology of the cell, leading to the improvement of cell adhesion in hypoxic EPCs (Fig. 4).

Collectively, our data provided evidence for the first time that hypoxia is associated with the impaired cell-matrix adhesion of EPCs via reduced mitochondrial function and biogenesis-dependent altered epigenetic regulation. However, exogenous NO administration restores the cell-matrix adhesion in cultured hypoxic EPCs via the miR-142-DNMT1/ITGA-5 axis. Further, our study is warranted for the development of a NO-based therapeutic strategy for the treatment of patients who suffers from high altitude hypoxia or tissue hypoxia-mediated EPCs dysfunction.

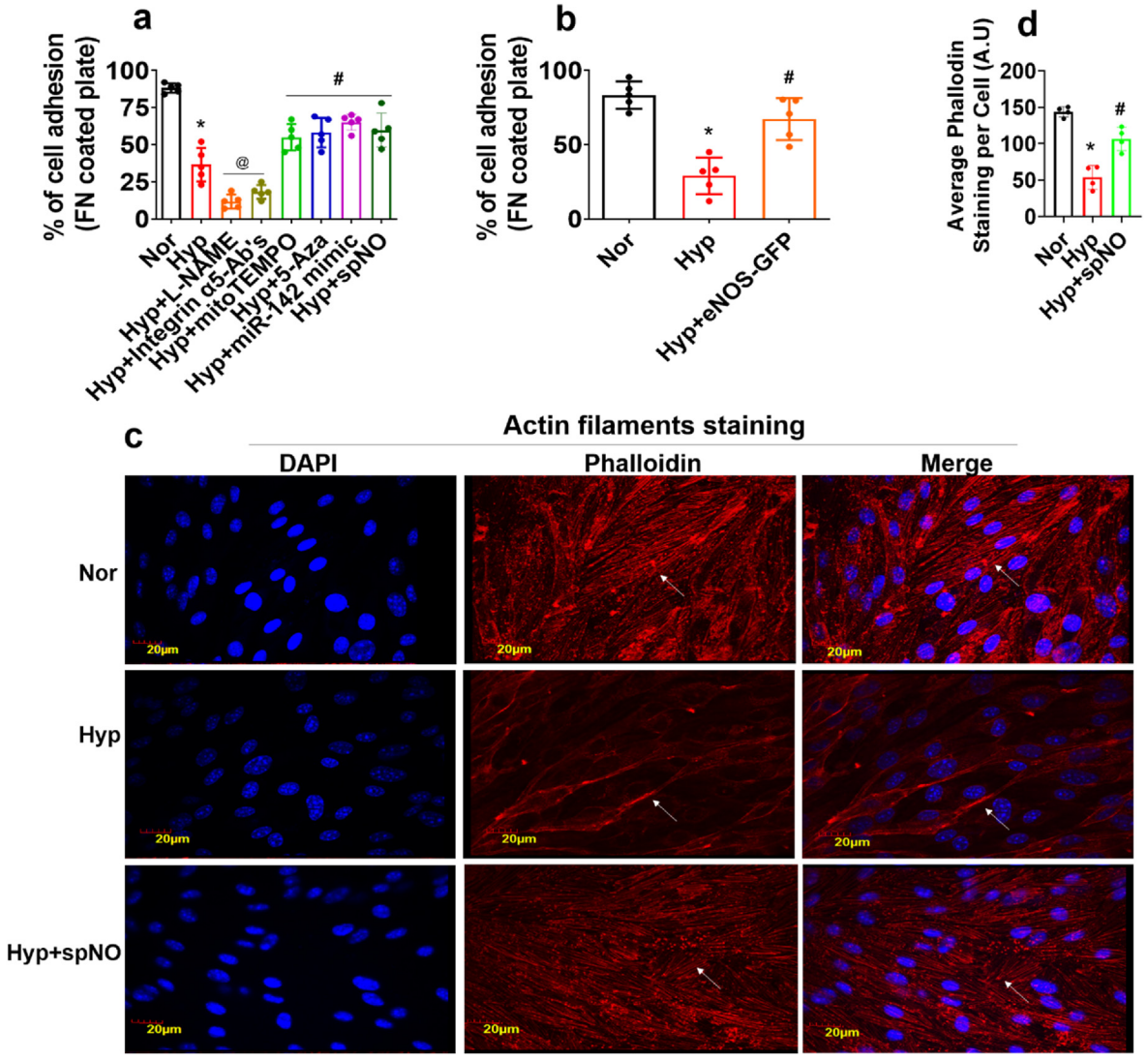

Fig. 4. ***Effect of NO and epigenetic inhibitor on cell-matrix adhesion in EPCs under hypoxia.*** *(a) Cell-matrix adhesion assay was performed in EPCs. (b, c) Cell-matrix adhesion assay in EPCs transfected with eNOS-GFP. (d, e) Phalloidin actin staining of cultured EPCs. Arrow head depicts the actin filaments staining. Data are expressed as mean ± SEM. n = 3-5 samples per group. *p<0.05 compared with the control Nor (normoxia), @p<0.05 compared with the Hyp (hypoxia) group, #p<0.05 compared with the Hyp (hypoxia) group. The error bars represent the s.e.m.*

Author contributions

JB and GS conceived the overall experimental idea and designed the framework for experimentation. JB conducted experimentation, analyzed and interpreted data, and wrote the manuscript draft. MSR provided expert opinion on data interpretation. GS supervised the overall project.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. G. Senguttuvan reports was provided by Anna University - Regional Office Tiruchirappalli. Senguttuvan Govindan reports a relationship with Anna University - Regional Office Tiruchirappalli that includes:.

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Abbreviations

ITGA-5	Integrin alpha-5
FITC	fluorescein isothiocyanate
DAPI	4′6-diamidino-2-phenylindole
DNMT1	DNA methyl transferase-1
SOD	Superoxide Dismutase
GPX	Glutathione Peroxidase
5-mC	5-methylcytosine
FN	fibronectin

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