이봄내린 석사학위논문

후기내피전구세포의 바이오마커 발굴 및 Hedgehog Interacting Protein 의 후기내피전구세포에서의 혈관 신생능 역할에 관한 연구

2016 년 7 월

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ABSTRACTS

The profiling of Biomarker In Late Endothelial Progenitor Cells and The role of Hedgehog Interacting Protein in Regulating Angiogenesis and Apoptosis Evasion

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The late endothelial progenitor cells (LEPCs) are derived from mononuclear cells (MNCs) and thought to directly incorporate into blood vessels and differentiate into mature Endothelial Cells (ECs). By transcriptome and proteome analysis, I identified distinctive LEPC profiles and found that Hedgehog-interacting protein (HIP) is strongly expressed in LEPC. Inhibition of HIP by lentiviral knockdown activated canonical hedgehog signaling in LEPC, while it activated non-canonical hedgehog signaling in ECs. In LEPC, HIP knockdown induced much enhanced tube formation and resistant to apoptosis in oxidative stress condition. While HIP is markedly expressed in proliferating LEPC, HIP expression is downregulated. during angiogenesis. Moreover HIP expression is reduced when angiogenic triggers such as VEGF and FGF2 are treated on LEPCs, My finding suggest that HIP regulate LEPC angiogenesis and survival via blocking canonical hedgehog signaling when there is no angiogenic stimulation.

Keywords: Late-Endothelial progenitor cell (LEPC), Hedgehog Interacting Protein (HIP), Angiogenesis, Hedgehog Signaling, Transcriptome analysis, Proteomics analysis

Student number: 2014-21927

CONTENTS

LISTS OF ABBREVIATIONS

HIP: Hedgehog Interacting Protein

LEPC: Late Endothelial Progenitor Cell

eEPC: Early Endothelial Progenitor Cell

Hh: Hedgehog

Shh: Sonic Hedgehog

EC: Endothelial Cell

MNC: Mononuclear Cell

HUVEC: Human Umbilical Vein Endothelial Cell

UCB: Umbilical Cord Blood

SCR: Scrambled

INTRODUCTION

Endothelial progenitor cells (EPCs) are circulating blood cells that are capable to promote vascular repair.[1] EPCs are derived from blood mononuclear cells (MNCs)[2] and play an essential part in coordinated postnatal vasculogenesis. Following injury to a tissue vasculature, hemostasis is initiated and it provide signal for mobilization and homing of circulating EPCs. EPCs migrated to damaged area divide, secrete cytokines that support angiogenesis and incorporate into vascular network to promote endothelial remodeling and neovasculogenesis.[1-3] In the presence of a stimulus such as a tumor, EPCs also play a major role in promoting tumor vasculature and supporting tumor growth.[4, 5]

EPCs are heterogeneous population; two subtype have been identified - early-EPCs (eEPCs) and late-EPCs (LEPCs). Various groups reported that LEPCs are proliferative subtype that form tube-like structure and directly incorporated into vasculature. On the other hand, eEPCs do not differentiate into mature endothelial cells (EC) and promote angiogenesis indirectly via paracrine manners.[6, 7] Based on these physiologic differences, only LEPCs are thought to give rise to mature endothelial cells followed by differentiation from Mononuclear cells (MNCs). Although the contribution of EPCs to ischemia induced or tumor angiogenesis is under intensive investigation, detailed molecular analysis of differentiation focusing on LEPCs is lacking.

Hedgehog (Hh) signaling has been gained attention as a key player in postnatal neovasculogenesis.[8, 9] In adult heart and skeletal muscle, sonic hedgehog (Shh) has been suggested to directly promote neovascularization and induce secretion of the pro-angiogenic growth factors. In tumor angiogenesis, Hh inhibition reduced tumoral VEGF secretion, reduced tumor vasculature and thereby renowned for anti-tumor effect.[10, 11] Moreover Hh ligands augments bone marrow-derived eEPC proliferation, migration and VEGF production by

Gli-1 dependent canonical Hh signaling.[12, 13] However in ECs, Hh proteins increase angiogenesis and migration through RhoA dependent non-canonical signaling pathways.[14, 15] Emerging evidence suggests that Hh signaling has central role in homeostasis and repair process by tightly regulating angiogenesis.

In this study, using high throughout RNA sequencing and mass spectrometry-based proteome analysis, I provide comprehensive approach into the characterization of endothelial lineage cells. This study is the first report of using both transcriptomic and proteomic approaches to clarify and characterize LEPCs in the context of physiologic differentiation stages. Unbiased expression profiling uncovers for the first time that Hedgehog Interacting Protein (HIP) is strongly expressed by LEPCs. HIP is a membrane glycoprotein that is known to inhibit Hedgehog signaling. Due to the striking role of Hedgehog signaling during developmental angiogenesis, I hypothesized that HIP play an important role in tight regulation of LEPC functions. Therefore, in this study, I investigated the role of HIP in regulation of LEPC angiogenesis.

MATERIALS AND METHODS

Cell culture

Late endothelial progenitor cells (LEPCs) and Early endothelial progenitor cells (eEPCs) were isolated from human umbilical cord blood as previously described[19]. The EPC isolation procedure was approved by Daegu Fatima Hospital and Seoul National University (IRB No. E1403/001-010). Human umbilical vein endothelial cells (HUVECs) were provided by by Dr. SM Kwon in College of Medicine, Pusan National University. LEPCs, eEPCs and HUVECs were maintained under a 5% CO_2 at mosphere in Endothelial cell growth medium (EGM-2 not including hydrocortisone) (Lonza) supplemented with 2% fetal bovine serum (FBS) on 1% gelatin coated dishes.

RNA-sequencing analysis

Total RNA was extracted from MNCs, MACs sorted LEPCs (CD146+ cells and CD117+ cells), and HUVECs using TRIzol Reagent (Life technology). The total RNA was treated with DNase I and then was purified with a miRNeasy Mini Kit (Qiagen). The quality of the RNA was checked with the Agilent 2100 Bioanalyzer (Agilent) prior to sequencing. Illumina platform for transcriptome with a 90-bp paired-end library (Illumina). Libraries were constructed following the Illumina Paired-End Sequencing Library Preparation Protocol. Library quality and concentration were determined using an Agilent 2100 BioAnalyzer (Agilent). Each sample was paired-end sequenced with Illumina HiSeq 2000 using HiSeq Sequencing kits.

Proteomics

Proteins was extracted from MNCs, MACs sorted LEPCs, and HUVECs using RIPA reagent (Thermo) according to the manufacturer's instruction. Quick StartTM Bradford 1x Dye Reagent (Bio-Rad Laboratories) was used to measure

protein concentration. Then, 120ug of proteins were loaded and each sample was prepared for 30 fractions (SDS-PAGE) that were further analyzed on LTQ-Orbitrap (Thermo Fisher) as previously described[20]. The datasets generated by LTQ-orbitrap were analyzed using Scaffold (version 4.4.1, Proteome Software Inc) Peptide identifications were accepted with 90.0% probability and FDR less than 1.0% by a Scaffold local FDR algorithm with at least 2 identified unique peptides.

shRNA Transfection and HIP overexpression vector construct

Human LEPCs were transfected with a specific HIP shRNA (sc-43835-SH) or control scrambled(SCR) shRNA (sc-108060), used as transfection control for 24 hours in EGM2 media. LEPCs were incubated 48 hrs after the transfection and Puromycin (50ng/ml) was added to select transfected LEPCs.

HIP Expression Vector Contruction

To create Hedgehog interacting protein (HIP) expression pcDNA3.1, HIP DNA was purchased from Korea Human Gene Bank (KRIBB). Human HIP was amplified using forward primer 5'-CGACTAGTTCTAGAATGCTGA AGATGCTCTCCTTTA and reverse primer 5'-GAGGGGCGGGATCCC TATACAATGTAACTTGTTAC. The amplified HIP gene was inserted into pcDNA3.1. eEPCs were transfected at day 6 of cell culture with pcDNA3.1 encoding full HIP gene or pcDNA3.1 without HIP for 24 hours in EGM2 media.

RT-PCR and Quantitative RT-PCR Analysis

Total RNA were extracted using Trizol reagent (Invitrogen) according to the manufacture's instruction. One μg of total RNA was used for cDNA synthesis with random hexamers using Omniscript RT kit (Qiagen) Primer and probe sequences are listed in the Supplemental Table. PCR was performed in a thermocycler (Biorad) with GoTaq® DNA polymerase (Promega). The relative expression of each mRNA was calculated by the comparative threshold cycle

(CT) method. One-way ANOVA was performed, and gene lists were created using a P value with a false discovery rate < 0.05

Tube formation assay

Tube formation assay on Matrigel was performed as described previously reported [21] In brief, culture plates were coated with 100 μl of growth factor-reduced Matrigel (BD Biosciences) per well. LEPCs seeded on coated plates at a density of 2.5×10^4 cells per Ill in EGM-2 plus 2% FBS in the presence of 20 ng/ml VEGF (R&D), folloId by an incubation at 37°C for 24 h. Tube formation was examined by light microscopy 12 h later.

Aortic Sprouting Assay

Mouse thoracic aortas were dissected from 6- to 8-week-old male C57BL/6 mice, As previously reported. The aortas were immediately transferred to Petri dishes and the adventitia and small vessels around the aorta were carefully removed. Aortas incubated in EGM2 containing lentivirus and polybrene (8ug/ml) for overnight. The resulting virus transfected aortic rings were embedded in 150 μL matrigel in EGM2 media with 20ng/ml VEGF in 96 well plate. The plates were incubated at 37°C in a humidified 10% CO2 atmosphere. Medium containing VEGF was replaced 3 times a week. After 14 days, micrographs of representative rings were taken and total number of vascular sprouts and branch points were calculated.

Migration Assay

Boyden chamber migration of LEPCs was performed as previously described [21]. In brief, LEPCs per well in 300 ul medium were added to the top chambers of 24-well transwell plates (5.0 ❍m, pore size; Costar). EGM-2 plus 2 % FBS in the presence 20 ng/ml of VEGF was added to the lower chambers. After 24 hours of incubation at 37 ℃ with 5 % CO2, the images were acquired on the bottom wells.

Western Blotting

Western blotting was performed as previously described[22] , cells were lysed in a RIPA buffer (thermor) with protease inhibitor cocktail (Roche). The supernatant of the lysates was collected and denatured by the same volume of SDS sample buffer (0.5 M Tris-HCl pH 6.8, 10% SDS, 50% glycerin, 2-mercaptoethanol). The protein samples were resolved in SDS-PAGE and blotted onto the PVDF membrane (Bio-Rad), which was then blocked with 5% bovine serum albumin for one hour. Next, Membranes were incubated with antibodies against Anti-HIP antibody (Abcam, ab39208), Anti-Gli-1 antibody (Biolegend, Cat642401), Anti-Cleaved Caspase-3 antibody (Cell signaling, Cat9661), Anti-VEGF antibody (Biolegend, 627501) at 4°C for twelve hours. Membranes were washed three times and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies for one hour. Blots were washed with TBST three times and developed with the ECL system (Thermo) according to the manufacturer's protocols..

Cell viability assay after oxidative stress

LEPCs were treated with H_2O_2 (100mM) for three hours. Then cells were incubated for one night and morphology was observed under a light microscope. Cell viability was measured by MTT assay (Sigma) to determined live cell growth after a day. One hundreds μl of 0.2mg/ml MTT was added to the media for five hours incubation at 37℃. Following removal of the culture medium, the remaining crystals were dissolved in 500 μl DMSO (Duksan) and absorbance at 470 nm was measured.

Statistical Analyses

Data are presented as means ± SD, and statistical comparisons between groups were performed by one-way analysis of ANOVA test. * symbol indicates $p<0.05$ vs. control group and + symbol indeicates $p<0.1$ vs. control group in one-way anova.

RESULTS

Human LEPCs Have Distinct Transcriptomic and Proteomic Profile

MNCs were isolated from human umbilical cord blood as previously described[23]. LEPCs were obtained by long term culture (14-21days) of MNCs. I then further isolated CD146-positive LEPCs and CD117-positive LEPCs to further purify LEPC population by magnetic cell sort analysis (MACS) separation. Mature vascular endothelial cells HUVEC were separated from the human umbilical vein of identical donor from which I collected cord blood. To investigate the molecular basis of stage specific differences, the four progenitor/mature cell populations were used as a source for both high throughout RNA-sequencing (RNA-seq) and semi-quantitative proteomics analysis (Fig. 1A). Robust and reproducible data were collected, with more than 42 million readings per population. In total, transcripts corresponding to 23,363 genes were identified (Fig. 1B). By performing proteome analysis using LC-ms/ms mass spectrometry, I identified a total of 2,127 proteins from all four samples (Fig. 1D).

A Pearson correlation analysis using genes differentially expressed among the four populations are consistent with differentiation hierarchy (Fig. 1C). PCA analysis revealed that MNCs are relatively heterogeneous cell types, while CD146-positive LEPCs, CD117-positive LEPCs and HUVECs are more mature and homogenous cell types. I found CD146 positive LEPCs and CD117 positive LEPCs are 99% alike, which indicate that EC and cardiomyocyte marker CD117 does not subdivide LEPCs at this initial differentiation stage. Data indicates that CD146 positive LEPCs and HUVECs share about 91% gene expression profiles.

Next, to systematically analyze the transcriptome and proteome at the transition from MNCs to CD146 positive LEPCs to HUVECs, I correlated the transcriptome data with proteome data. I found 1,947 proteins out of 2,127 proteins from proteome data were correlated to the transcriptome data.

To identify a stage-selective LEPC markers and endothelial markers, I compared 2-fold upregulated genes found both in transcriptome and proteome data set. I found 244 genes were upregulated in LEPCs compared to MNC in both RNA and protein levels. Next, I compared HUVECs and MNCs. I found 164 genes were upregulated in HUVECs compared to MNCs in both transcriptome and proteome data set. Finally I found 101 genes common in 244 and 164 genes enriched in endothelial lineage compared to MNCs. EC signature genes are provided in supplementary data1. These genes could serve as endothelial lineage signature genes involved in endothelial commitment process (Fig. 1E).

To gain insight into the commitment process, I performed gene ontology (GO) analysis. Gene ontology (GO) analysis indicated that upregulated genes in MNCs mainly related in immune response and cell activation including T cell, leukocyte and lymphocyte activation (Fig. 1G). The 101 upregulated genes in endothelial commitment mainly participated in cell adhesion, cytoskeleton organization and cell motion (Fig. 1F). This is partly due to the nature of endothelial commitment and differentiation from the circulating blood MNC since it requires coordinated multistep processes including mobilization, adhesion, transmigration and incorporation. Uniquely enriched genes of CD146 positive LEPCs compared to HUVEC participated in chromosome organization, DNA replication and chromatin assembly (Fig. 1H).

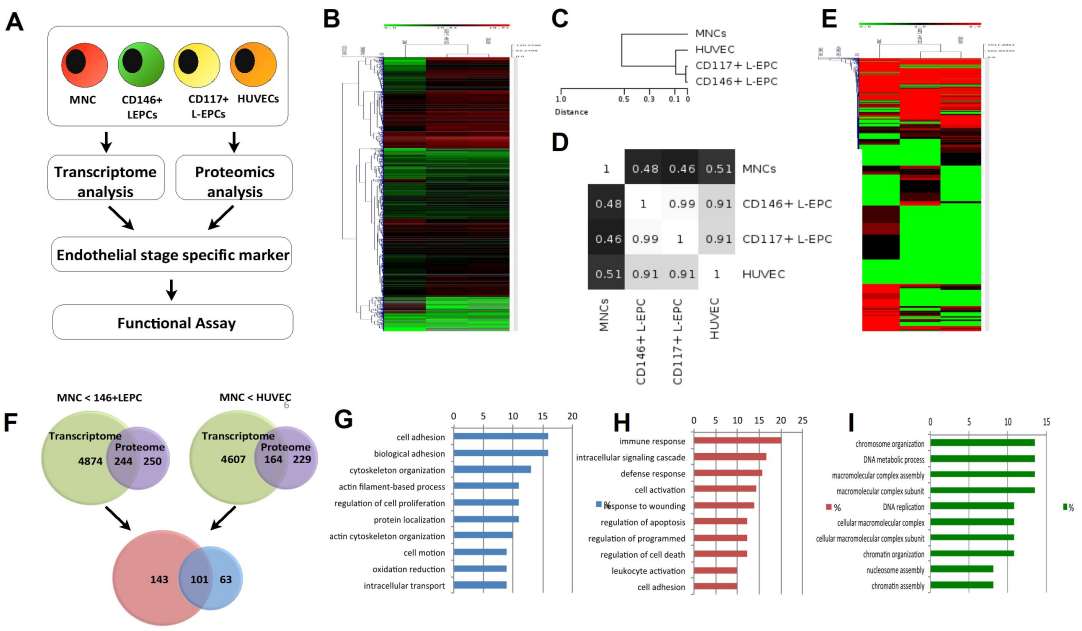


Figure 1. Transcriptome and proteome analysis of MNCs, CD146 positive LEPCs, CD117 positive LEPCs and HUVECs. **(A)** Experimental design used to identify endothelial lineage selective molecular signatures. **(B)** Heat map demonstrating differential gene expression. Since CD146 positive LEPCs and CD117 positive LEPCs are 99% alike. CD117 results are not shown. The figure was created using the Mev software. Red and green colors indicate upregulated and downregulated genes respectively. Genes were further grouped using hierarchical clustering (distance metrics was Euclidean distance and linkage method was average). **(C)** Transcriptome differences between MNCs, CD146, CD117-positive LEPCs, and HUVECs in distance. **(D)** Pearson correlation test for the four cells, MNCs, CD146, CD117-positive LEPCs and HUVECs. **(E)** Heat map demonstrating differential protein expression. **(F)** Overlapped 244 genes shown are the ones upregulated at the RNA and protein level in CD146+ LEPC compared to MNCs. Overlapped 164 genes are upregulated in HUVEC compared to MNCS. 101 genes overlapped in the lower data indicated endothelial signature genes which are upregulated in both CD 146+ LEPCs and HUVECs compared to MNCs. **(G)** The top 10 overrepresented biological processes of endothelial signature genes in GO analysis. **(H)** The top 10 overrepresented biological

process of MNC signature genes in GO analysis. MNC signature genes are upregulated genes in MNCs compared to both LEPCs and HUVECs. (I) The top 10 overrepresented biological process of CD146+ LEPC stage signature genes compared to HUVEC.

HIP is Selectively Expressed in LEPCs and ECs

Among 101 enriched genes in endothelial lineage compared to MNCs, I focused on HIP based on Hh (Hedgehog) signaling's importance during developmental angiogenesis. HIP transcripts have been confirmed by quantitative real-time RT-PCR and western blot. In MNC and eEPCs, HIP was very low in transcript and not detected in protein level (Fig. 2A & 2E). As both Hh antagonist HIP and Hh receptor Ptc-1 are transcriptional targets of Hh signaling I examined the expression levels of Sonic hedgehog (Shh) and Ptch-1. Interestingly Ptch-1 expression was similar in MNCs, eEPCs, LEPCs and HUVEC, while HIP expression patterns were reversely correlated to Shh expression pattern among eEPC, LEPC and HUVEC (Fig. 2C & 2D).

Previously, it was shown that HIP is downregulated during active angiogenesis. I tested the expression of HIP during tube-formation. Indeed, the expression of HIP mRNA extracted from the cells of tube formed on Matrigel was 2.8-fold lower. Moreover canonical Hh target Gli1 expression was 48 fold upregulated in the tube forming, HIP-lower cells (Fig. 2F & 2G). As it is well described that various cytokines actively promote angiogenesis and tube formation, it is conceivable that HIP is down-regulated by growth factors that promote angiogenesis. Thus I measured HIP mRNA after treating angiogenic growth factors in LEPCs. I observed that VEGF and FGF2 downregulated significantly HIP mRNA expressions (Fig. 2H & 2I). The downregulation effect was more prominent after treating FGF2 than VEGF with same concentrations.

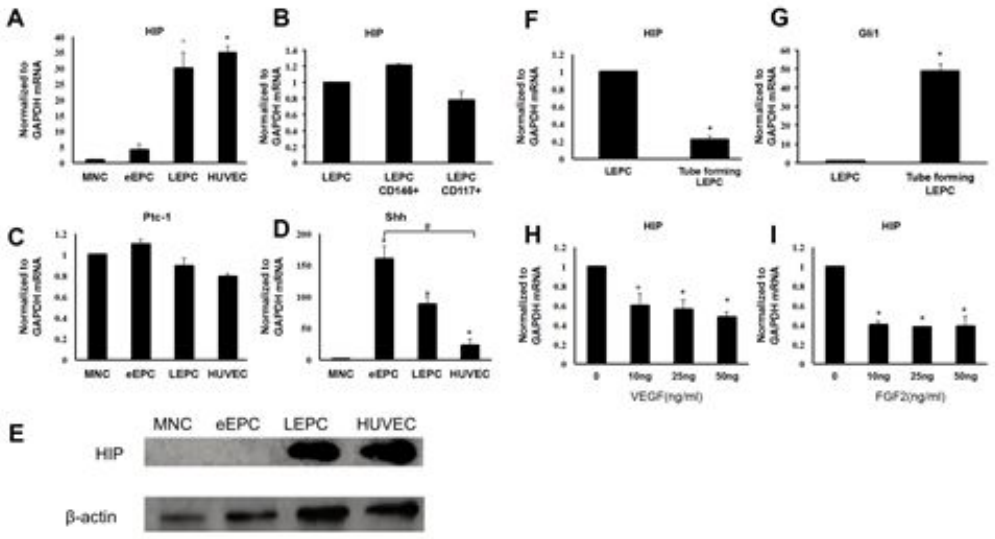


Figure. 2 Hedgehog interacting protein is selectively expressed in late endothelial progenitor cells.

Human mononuclear cells and early endothelial progenitor cells were isolated and cultured on fibronectin for 7 days. **(A)** Expression levels of HIP in MNC, eEPC, LEPC and HUVEC assessed by Q-RT-PCR. *P<0.05 vs. MNC **(B)** Expression levels of HIP in LEPC, CD146 positive LEPC andCD117 positive LEPC **(E)** Expression level of HIP protein in MNC, eEPC, LEPC and HUVEC. **(C) - (D)** Ptc-1 and Shh mRNA expression level in MNC, eEPC, LEPC and HUVEC *P<0.05 vs. MNC, #P<0.05 vs. eEPC **(F)-(G)** HIP and Gli1 mRNA expression under in vitro angiogenic conditions on Matrigel was measured by Q-RT-PCR. *P<0.05 vs. LEPC **(H)-(I)** LEPCs were treated with FGF2 or VEGF for 16 hrs and HIP mRNA expression was measured by qRT-PCR. *P<0.05 vs. LEPC unstimulated

HIP knockdown Enhanced LEPC Angiogenesis and Mouse Aortic Sprouting

To determine the role of HIP in LEPC, I generated lentiviral based HIP shRNA (shHIP). HIP knockdown was confirmed in RNA and protein level (Fig

.3A & 3B). The effects of HIP knockdown was investigated in capillary morphogenesis assay. Capillary morphogenesis assay indicated that HIP knockdown increased the number of tube formed on Matrigel (Fig. 3C). I found that HIP inhibited LEPC formed more durable tubes that existed longer (Fig. 3D). Next, to investigate the functional significance of HIP inhibition in vivo, I assessed aortic sprouting capacity in mice. Thoracic artery was dissected and transfected with shHIP lentivirus and seeded on Matrigel. Fig 3F demonstrated that knockdown of HIP enhanced aortic sprouting Total number of vascular sprouting and branching points was increased more than 50% as compared with scrambled lentivirus transfected aorta (Fig. 3E & F). These finding cleary indicate the functionl relevance of HIP knockdown for enhanced LEPC angiogenesis and newly sprouting aorta.

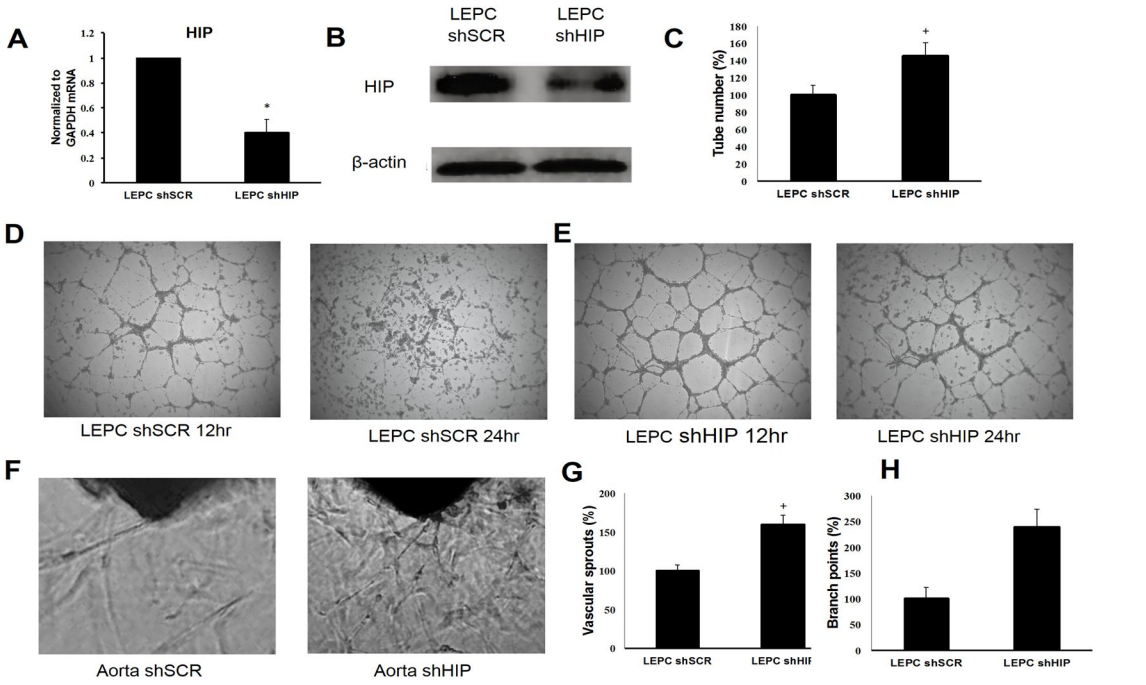


Figure 3. Inhibition of HIP in LEPCs enhances angiogenesis **(A)-(B)** Lentiviral shHIP knockdown vector and scramble vector were treated to LEPC for overnight and after 48 hrs incubation downregulated HIP level was confirmed by qRT-PCR and Western blot. *P<0.05 vs. LEPC shSCR **(C)-(E)** LEPCs were suspended and cultured on Matrigel with 20 ng/ml VEGF. After 12 hrs and 24

hrs later, (C) tube number was counted and (D,E) tube formation was assessed under light microscopy (F)-(H) Mouse thoracic aortas are dissected and transfected with ship knockdown vector and scramble vector. Aortas are then seeded on Matrigel containing 20ng/ml VEGF. After 10 days, (F) sprouting aortas were measured on microscope and (G) vascular sprouts number and (H) branch points were calculated.

HIP Knockdown Decreased Apoptosis in Oxidative Stressed LEPCs.

Since LEPCs are faced with oxygen deprivation in ischemic sites where LEPCs are actively involved in angiogenesis, I hypothesized that HIP inhibition will increase resistance to apoptosis and exhibit decreased proteolytic activation of caspase-3. After the treatment of 100mM H_2O_2 to LEPC for 3 hours, cells were washed out, incubated overnight and viable cells were measured, detected via DAPI staining and MTT assay (Fig. 4A-C). LEPCs with low HIP were less susceptible to oxidative stress than normal LEPCs upon treatment of H_2O_2. Moreover, LEPCs with low HIP showed decreased caspase-3 cleavage activity than normal LEPCs after the treatment of H_2O_2 (Fig. 4G). Without oxidative stress I measured LEPC proliferation. Interestingly inhibition of HIP did not affect LEPC's proliferation and cell cycle regulators, cyclin D and E (Supplementary Fig. 1). Next, I hypnotized that HIP knockdown affect LEPC migration and invasion, since these are important character of LEPC in ischemic injury. However migration and wound closure remained unchanged with HIP inhibition.

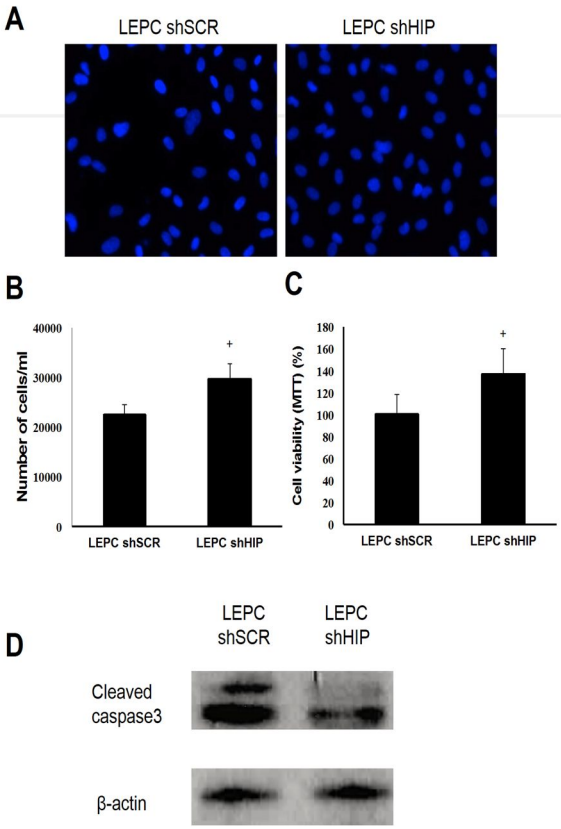
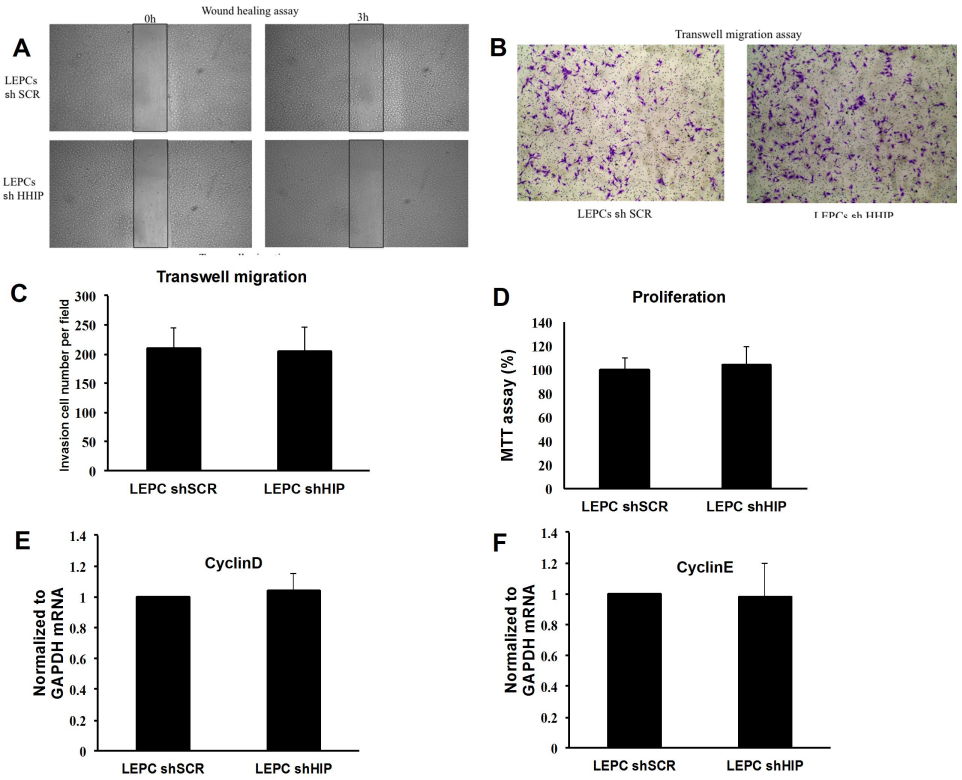


Figure 4. Knockdown of HIP enhances survival and viability of LEPC upon oxidative stress

LEPCs were treated with 50 mM Hydrogen peroxide for 3 hours and cell survival and viability was assessed After hydrogen peroxide induced oxidative injury in LEPC, **(A)** cells were incubated overnight and stained with DAPI , **(B)** total number of cells/ml was calculated and **(C)** MTT assay was performed. +P<0.1 vs. LEPC shSCR **(D)** Cleaved caspase 3 level was measured by Western blot .



Supplementary Figure 1. Inhibition of HIP does not affect LEPC migration and proliferation.

(A) LEPC with low HIP migration was assessed by scratch (wound healing) assay. **(B)** LEPC with low HIP migration was visualized by trans-well migration assay. **(C)** Transwell invasion cell number per/field was calculated **(D)** LEPC with low HIP proliferation was assessed by MTT assay. **(E)-(F)** Cyclin D and Cyclin E expression was measured by Q-RT-PCR after knockdown of HIP.

Hedgehog Protein Activates Canonical Hh Signaling in LEPCs

It has been known that canonical Hh signaling is activated in eEPC while non-canonical signaling is activated in EC when treated with Hh ligands. However, exact Hh response in LEPC has not been studied. Moreover since it has been thought that LEPCs might be just a detached cells from vessel wall,

molecular differences between LEPCs and HUVEC have not been studied well. I originally hypothesized that, like HUVEC, non-canonical Hh signaling mediates the Ptc-1 activation in LEPCs. Since there has been no study on this, I examined the effect of Hh ligands on LEPC. LEPCs were treated with Shh and examined the expression of canonical and non-canonical Hh target genes by qRT-PCR. Interestingly, I found that canonical Hh targets including Gli-1, VEGFA, Ang1, PTC-1 and HIP were upregulated upon treated with Shh, while OPN and MMP2, non-canonical target genes, showed relatively unchanged expression (Fig. 5A-F). On the contrary, as reported, HUVECs showed the induction of non-canonical Hh targets OPN and MMP2 upon Shh treatment without Gli-1 increase (Fig. 5G-3J). western blot results confirmed that Gli-1 expression was enhanced upon after Shh treatment in LEPC while Shh treatment yielded no change in HUVEC (Fig. 5K). Together these results suggests that LEPCs and ECs respond differently to Hh ligands and canonical Hh signaling plays an important role in LEPCs. In figure. 5L, LEPCs and HUVECs showed both enhanced tube formation with 200ng/ml Sonic hedgehog protein. We observed that LEPCs treated with Shh showed more enhanced angiogenesis than HUVEC treated with Shh. This could be due to the different signaling mechanism although non-canonical Hh signaling seems also play a role in angiogenesis, canonical Hh signaling is known to induce strong angiogenesis.

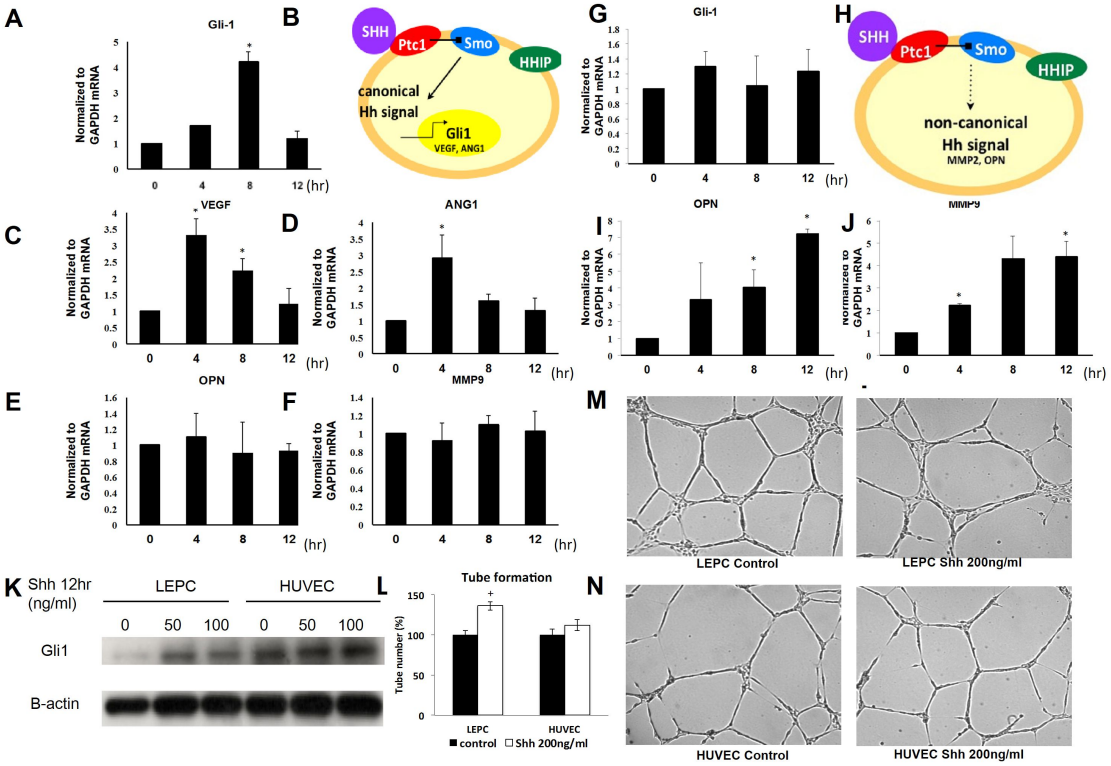
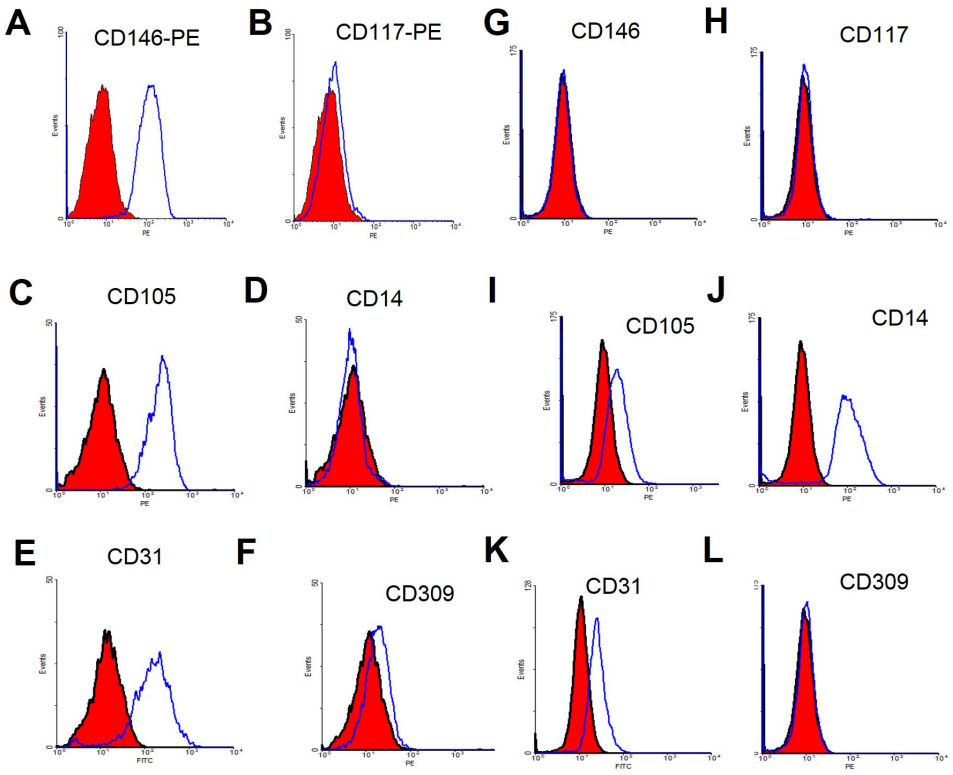


Figure 5. Shh significantly activates Hedgehog signaling via canonical Gli-1 dependent pathway in LEPCs.

(A)-(F) LEPCs were cultured with 100 ng/ml Shh for 0, 4, 8, 12 hours and then canonical Hh target genes including Gli-1, Ptc-1, VEGFA, and ANG1, and non-canonical Hh signal target genes including OPN and MMP2 were analyzed by qRT-PCR. *P<0.05 vs. LEPC unstmulated. **(G)-(J)** HUVECs were cultured with 100 ng/ml Shh for 0, 4, 8, 12 hours and then Gli-1, OPN and MMP2 were analyzed. *P<0.05 vs. HUVEC unstimulated **(K)** LEPCs and HUVECs were cultured with 0 ,50, 100 ng/ml Shh for 12 hrs and then Gli-1 expression was assessed by western blot analysis. **(L)** LEPCs and HUVECs were induced tubeformation with 0 (control) or 200ng/ml Shh for 12 hours and tube numbers were measured. (M) Tube formation of LEPCs control (unstimulated) and LEPC treated with 200ng/ml Shh **(N)** Tube formation of HUVECs control (unstimulated) and LEPC treated with 200ng/ml Shh

HIP Overexpression in eEPC Reduced Paracrine Effects on ECs

It has been reported that different roles of early and late EPC in vasculogenesis contributed equally to neovasculogenesis in vivo. The eEPCs contribute angiogenesis mainly by secreting cytokines that support resident mature EC's angiogenic function. Therefore, I tested the role of HIP in eEPCs in regards to angiogenesis. eEPCs used for the experiments were first characterized via FACs staining (Supplementary Fig. 2). Since I demonstrated that HIP knockdown activates canonical Hh signaling and VEGFA expression in LEPCs, I tested the hypothesis that increased HIP expression in eEPC reduces the VEGFA expression and release into the extracellular space would lead to subsequent regulation of mature EC function (Fig. 6). First, I treated HIP overexpressing lentiviral vector to eEPCs and confirmed the overexpression level in eEPCs. More than 15-fold of HIP overexpression was detected and subsequent low expression of Gli-1 was detected in eEPC by qRT-PCR. Second, I verified the effect of conditioned media of eEPCs on HUVEC; HUVECs treated with the conditioned media of eEPC which has HIP overexpression showed reduced tube formation. Third, I extracted RNA from the HUVECs treated with the conditioned media of eEPC which has HIP overexpression for two days. Compared to the HUVECs treated with the conditioned media from normal eEPC, HUVECs treated with the media of eEPC with high HIP showed reduced expression of VEGFR2 which is a direct downstream pathway of VEGFA signaling.



Supplementary Figure 2. Flow cytometry analysis of LEPC and eEPC

The overlaid histograms of analyzed markers with their unstained control.

(A)-(F) Expression of markers in LEPC.

(G)-(L) Expression of markers in eEPCs.

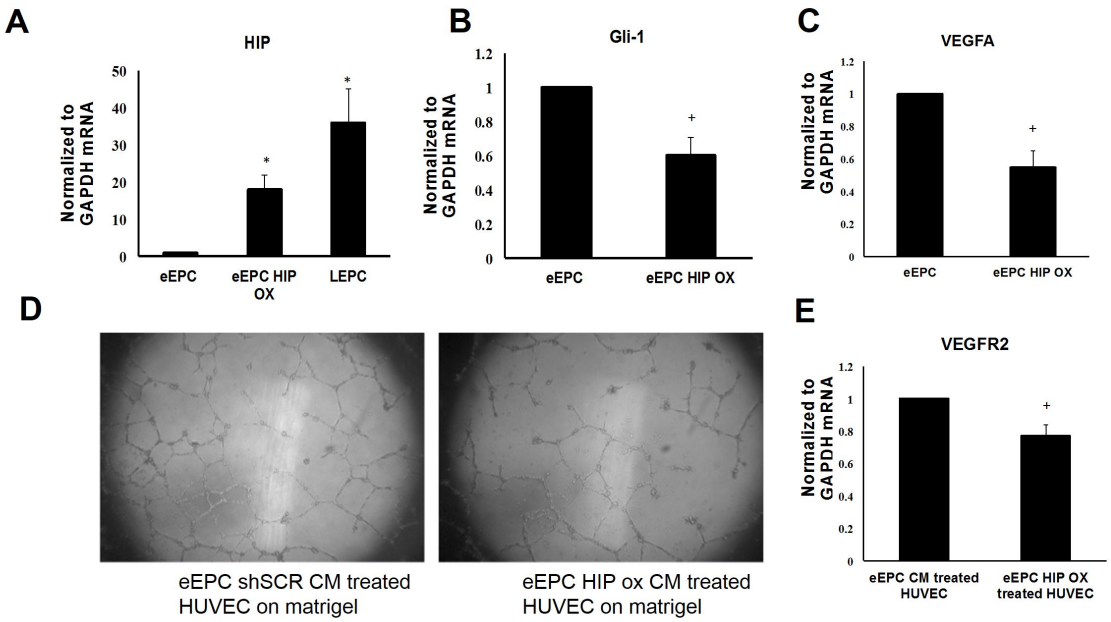


Figure 6. Overexpression of HIP in eEPCs impedes its paracrine angiogenic effect on EC. The eEPCs were cultured for 6 days and transfected with HIP overexpression vector for 24 hrs. After transfection eEPCs were incubated in Endothelial basal media without growth factor and with 2% FBS. **(A)-(C)** HIP, Gli-1 and VEGFA expression was measured by qRT-PCR. *$P<0.05$ vs. eEPC, +$P<0.1$ vs. eEPC **(D)** Conditioned medium from eEPC HIP overexpressed or eEPC were treated to HUVEC suspended on Matrigel for 12 hrs. Tube formation was visualized. **(E)** VEGFR2 level of conditioned medium treated HUVEC was assessed. +$P<0.1$ vs. eEPC CM treated HUVEC

HIP Knockdown Enhanced Angiogenesis and Apoptosis Evasion of LEPC Through Activation of the Canonical Hh signaling

Since I demonstrated that canonical Hh signaling is activated upon Shh stimulation in LEPCs, I hypothesized that HIP function to block canonical Hh signaling in LEPCs. Indeed, I found that inhibition of HIP enhanced Gli-1 protein expression (Fig. 7A). Upon Shh treatment, Gli-1 mRNA is expressed at a high level in eEPCs while it is low in LEPCs. This result suggests that high

expression of HIP may inhibit Hh signaling in LEPCs since their Ptch-1 expression was similar. Thus, when I treated LEPC with Shh after knocking down the HIP, the Gli-1 expression was as high as the eEPC with Shh simulation, suggesting that inhibition of HIP enhanced Hh signal responsiveness to Shh treatment (Fig. 7B). These results suggest high level of HIP in LEPC even with high level of Ptc-1 receptor efficiently block the canonical Hh signaling upon Shh stimulation. To further test the casual relations between HIP and canonical Hh target genes, I used specific pharmacological Hh inhibitors, Cyclopamine and GANT61. I found that the enhanced Gli-1 expression by of HIP knockdown was abolished by both Smo inhibitor Cyclopamine and Gli inhibitor GANT61 (Fig. 7C & 7D). As results, I found that enhanced angiogenesis and the reduced apoptosis after oxidative stress with HIP knockdown were completely abolished by GANT61 in LEPC (Fig. 7E & 7F).

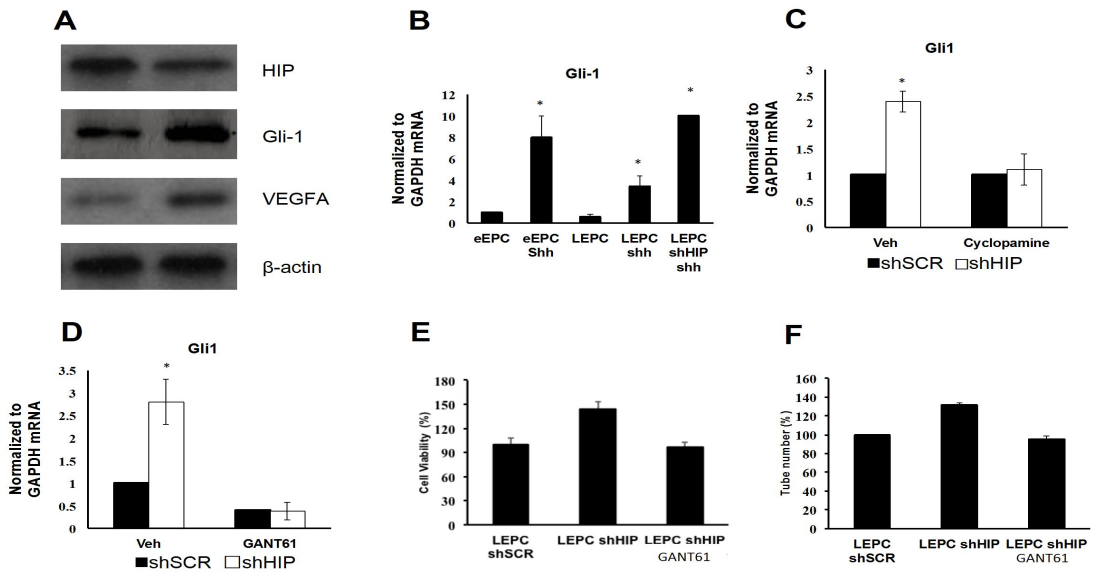


Figure 7. HIP knockdown activates canonical Hh target genes and enhances Hh responsiveness. **(A)** Inhibition of HIP increased Gli-1 and VEGFA expressions. **(B)** Shh 100 ng/ml were treated for 12 hrs in eEPC, LEPC and LEPC with low HIP expression and then Gli-1 activation was assessed by qRT-PCR. *P<0.05 vs. eEPC **(C)-(D)** LEPCs and LEPCs with low HIP were treated with vehicle

(DMSO), Hh inhibitor Cyclopamine (20 uM) or Gli1 specific inhibitor GANT61 (20 uM) for 12 hrs and Gli1 expression was measured. *P<0.05 vs. Veh shSCR **(E)** After treatment of 20 uM GANT61 for 24 hours, LEPCs with scramble and LEPC with HIP knockdown were detached and seeded on Matrigel with 10 ng/ml VEGF. After 12 hrs, tube number was measured. **(F)** After treatment of 20 uM GANT61 for 24 hours, LEPCs with scramble and LEPC with HIP knockdown were treated with 50 uM H_2O_2 for 3hrs and MTT assay was performed.

DISCUSSION

In the present study, we demonstrated HIP is an important regulator of early and late EPCs function in angiogenesis. We showed, for the first time to our knowledge, that HIP is expressed in LEPCs. Inhibition of HIP increases LEPC tube formation and resistant to oxidative stress. Moreover since there have been no precise molecular mechanism how Hh pathway could contribute LEPC function. In this regard, we demonstrated Shh activate the Gli-1 dependent canonical Hh pathway in LEPCs and thus, Inhibition of Hip affect LEPCs function via canonical Hh signaling. In addition, overexpression of HIP regulates paracrine function of eEPCs, which leads to decreased EC tube formation. Finally we demonstrated that HIP expression is decreased in LEPC upon treated with VEGF, FGF2 or seeded on Matrigel. Reduced HIP expression in such angiogenic trigger suggests that EPC function mediated by Hh signaling is tightly regulated and initiated by angiogenic stimulus.

Previous investigations indicate that HIP is highly expressed in adult heart, lung, brain, kidney and testis. HIP expression is decreased in several human tumors of the lung, stomach, colorectal tract, and liver compared with the corresponding normal tissues. It was reported that HIP is epigenetically inactivated by hypermethylation.[41, 42] As its silencing enhanced Hh signaling actively involved in tumor growth and survival, it was suggested that stromal cells expressing HIP regulate the proliferation of several tumor and various tumor actively downregulated HIP expression.[40]

However, the precise regulation mechanism of HIP in adult tissue has been poorly understood. Sekiguchi et al. described that estradiol triggers Shh induced angiogenesis during peripheral nerve regeneration by downregulating HIP.[43] J Coulombe et al. suggested the soluble form of HIP exist in rodent brain.[44] Holtz et al. reported that HIP non-cell autonomously inhibits Hh dependent neural progenitor patterning and proliferation.[45] These emerging evidences suggest that HIP functions as important inhibitor that timely regulates adult stem cell function and differentiation. In this regard, we found that HIP is upregulated during blood monocyte differentiation into endothelial lineage and it is silenced with angiogenetic triggers. Previous investigation indicate that Shh, VEGF and FGF2 are upregulated in ischemic tissue and injury.[2] While Hh receptor Ptch-1 remained relatively unchanged, it suggests that HIP is an important regulator that tightly regulate EPC differentiation and angiogenesis when it is requested by angiogenic trigger.

The vascular endothelium line the entire circulatory system and endothelial cells turn over very slowly to remain homeostasis. Only following ischemic disease or injury, rapid proliferation and angiogenesis initiated. In this regard, we suggest that HIP functions to restrain EPC at steady state. Only with proper angiogenic trigger, Hip expression was reduced and inhibition of HIP greatly affect LEPC angiogenesis and resistant to oxidative stress.

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ABSTRACT IN KOREAN

후기내피전구세포는 내피전구세포의 일종으로 단핵구로부터 유래하며 혈관을 신생하는 성체줄기세포의 일종이다. 순환중인 후기내피전구세포는 혈관에 상처나 허혈이 생겼을 때 직접적으로 혈관에 부착하여 혈관형성을 돕고 내피세포로 분화된다. 이번 연구에서는 단핵구, 후기내피전구세포, 내피세포간의 전사체분석과 프로테오믹스 분석을 통해 줄기세포 치료에 응용할 수 있는 후기내피전구세포의 특징적인 마커를 찾고자 하였다. 그 결과 Hedgehog Interacting Protein (HIP) 이 후기내피전구세포에 특징적으로 발현함을 확인하였다. Hedgehog Interacting Protein (HIP)은 세포 표면 단백질로 Hedgehog signal을 제한하는 유일한 표면 길항 단백질이다. Lentivirus를 이용해 HIP 발현을 억제시켜 후기내피전구세포에서의 역할을 살펴본 결과, HIP은 후기내피전구세포에서 혈관신생과 세포사멸 억제 효과를 저해하고 있음을 확인하였다. 또한 내피전구세포의 다른 일종인 초기내피전구세포에서 HIP의 발현을 유도시켰을 때는 초기내피전구세포의 특징적인 간접적인 혈관신생 촉진 기능이 사라짐을 확인하였다. 이러한 효과들은 성숙한 혈관세포에서와 다르게 canonical Hedgehog signal을 저해함으로써 나타남을 확인하였다. 또한 HIP의 발현은 Matrigel 위해서 후기내피전구세포의 혈관신생이 유도되었을 때나, VEGF, FGF2와 같은 혈관신생을 촉진하는 cytokine을 주입했을 때 확연히 떨어짐을 확인하였다. 따라서 HIP의 기능은 혈관신생이 직접적인 cytokine과 같은 촉발제가 없는 일반적인 생리 상황에서 혈관신생이 시작되지 않도록 억제하는 역할을 하고 있는 것으로 기대된다.