Identification and Functional Characterization of Hypoxia-Induced Endoplasmic Reticulum Stress Regulating IncRNA (HypERlnc) in Pericytes

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Rationale: Pericytes are essential for vessel maturation and endothelial barrier function. Long noncoding RNAs regulate many cellular functions, but their role in pericyte biology remains unexplored.

Objective: Here, we investigate the effect of hypoxia-induced endoplasmic reticulum stress regulating long noncoding RNAs (HypERlnc, also known as ENSG00000262454) on pericyte function in vitro and its regulation in human heart failure and idiopathic pulmonary arterial hypertension.

Methods and Results: RNA sequencing in human primary pericytes identified hypoxia-regulated long noncoding RNAs, including HypERlnc. Silencing of HypERlnc decreased cell viability and proliferation and resulted in pericyte dedifferentiation, which went along with increased endothelial permeability in cocultures consisting of human primary pericyte and human coronary microvascular endothelial cells. Consistently, Cas9-based transscriptional activation of HypERlnc was associated with increased expression of pericyte marker genes. Moreover, HypERlnc knockdown reduced endothelial-pericyte recruitment in Matrigel assays (P<0.05). Mechanistically, transcription factor reporter arrays demonstrated that endoplasmic reticulum stress-related transcription factors were prominently activated by HypERlnc knockdown, which was confirmed by immunoblotting for the endoplasmic reticulum stress markers IRE1α (P<0.001), ATF6 (P<0.01), and soluble BIP (P<0.001). Kyoto encyclopedia of genes and gene ontology pathway analyses of RNA sequencing experiments after HypERlnc knockdown indicate a role in cardiovascular disease states. Indeed, HypERlnc expression was significantly reduced in human cardiac tissue from patients with heart failure (P<0.05; n=19) compared with controls. In addition, HypERlnc expression significantly correlated with pericyte markers in human lungs derived from patients diagnosed with idiopathic pulmonary arterial hypertension and from donor lungs (n=14).

Conclusions: Here, we show that HypERlnc regulates human pericyte function and the endoplasmic reticulum stress response. In addition, RNA sequencing analyses in conjunction with reduced expression of HypERlnc in heart failure and correlation with pericyte markers in idiopathic pulmonary arterial hypertension indicate a role of HypERlnc in human cardiopulmonary disease. (Circ Res. 2017;121:368-375. DOI: 10.1161/CIRCRESAHA.116.310531.)

Key Words: cardiovascular diseases ■ heart diseases ■ pericytes ■ pulmonary heart disease ■ RNA, long noncoding
Pericytes are perivascular mural cells that contribute to endothelial maturation and vessel stability at the level of the microvasculature. Pericyte recruitment toward the vessel wall is mainly driven by the platelet-derived growth factor signaling axis. In vivo, a block of platelet-derived growth factor signaling results in pericyte death and impaired pericyte recruitment toward endothelial cells resulting in endothelial dysfunction, including endothelial barrier breakdown. These events facilitate extravasation of macromolecules with subsequent inflammation and organ remodeling. In organ injury and fibrotic disease, pericytes may hyperproliferate and contribute to wound healing and organ remodeling. Despite their important role within the cardiovascular system and various disease states such as idiopathic pulmonary arterial hypertension, molecular mechanisms that control human pericyte (hPC) survival and differentiation are poorly understood.

### Novelty and Significance

**What Is Known?**
- Pericytes are essential perivascular cells that induce vessel maturation, stabilize endothelial barrier function, and contribute to organ remodeling in pathological conditions. Pericyte survival and recruitment toward endothelial cells are mainly driven by platelet-derived growth factor signaling; however molecular regulatory mechanisms that control pericyte differentiation and survival are poorly understood.
- Long noncoding RNAs represent noncoding transcripts that have been found to significantly regulate endothelial as well as smooth muscle cell function, but their role in pericyte biology remains unclear.

**What New Information Does This Article Contribute?**
- Here, we characterize hypoxia-regulated long noncoding RNAs in pericytes and show that HypERlnc significantly regulates human pericyte function, differentiation, and survival by modulating the endoplasmic reticulum (ER) stress response.

**Long noncoding RNAs (lncRNAs) represent a large proportion of the noncoding transcriptome and act by various mechanisms that affect transcriptional and epigenetic control of gene expression. In addition, it is well documented that lncRNAs can regulate post-transcriptional processes such as splicing. Previous studies have shown a regulatory role of lncRNAs in endothelial and smooth muscle cells. For example, IncRNA MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) has been shown to regulate endothelial cell function, whereas lncRNA SENCR (smooth muscle and endothelial cell-enriched migration/differentiation-associated long noncoding RNA) has been demonstrated to stabilize the smooth muscle cell contractile phenotype. Recent experimental studies have outlined the importance of lncRNAs in modulating clinically relevant processes such as cholesterol synthesis and cardiac hypertrophy, making them promising molecular targets in cardiovascular disease. However, the contribution of lncRNAs to hPC function is yet unclear.

Pericytes are mural cells that contribute to vessel maturation and control endothelial barrier function. Despite their pivotal role in the cardiovascular system, knowledge is sparse on molecular regulatory mechanisms of pericyte cell biology. Here, we show for the first time that a long noncoding RNA is essential for pericycle function, proliferation, and differentiation. Our findings on the regulation of ER stress by HypERlnc may have a broad translational impact. ER stress has been shown to be involved in various disease states, including heart failure. Our findings that HypERlnc is significantly deregulated in human heart failure and significantly correlates with heart failure. Our findings that HypERlnc may have a role in human cardiopulmonary disease. The identification of a HypERlnc orthologue in mouse pericytes will enable to perform translational studies that may substantiate these findings in vivo and elucidate the role of HypERlnc in cardiopulmonary disease.

### Methods

**Characterization of hPCs**

RNA sequencing (RNA-seq) of hPC in normoxic and hypoxic conditions demonstrates robust expression of pericyte markers (eg, platelet-derived growth factor receptor-β and NG2), which was confirmed by immunoblotting (Online Figure I). In addition, hPC formed functional intercellular junctions as indicated by intercellular dye transfer live cell imaging experiments with human umbilical vein endothelial cell (Online Figure II), suggesting that the cells in the present study are indeed pericytes.

**HypERlnc Is Induced by Hypoxia and Is Expressed in the Nucleus and Cytosol of hPCs**

To detect regulatory IncRNAs in hPC, they were subjected to 24 hours of hypoxia followed by RNA-seq. Along with known hypoxia-regulated transcripts (eg, H19 and MIR210HG)

### Analyses of HypERlnc in Human Hearts and Lungs Suggest That HypERlnc May Have a Role in Cardiopulmonary Disease States Such as Heart Failure and Idiopathic Pulmonary Hypertension.

Pericytes are mural cells that contribute to vessel maturation and control endothelial barrier function. Despite their pivotal role in the cardiovascular system, knowledge is sparse on molecular regulatory mechanisms of pericyte cell biology. Here, we show for the first time that a long noncoding RNA is essential for pericycle function, proliferation, and differentiation. Our findings on the regulation of ER stress by HypERlnc may have a broad translational impact. ER stress has been shown to be involved in various disease states, including heart failure. Our findings that HypERlnc is significantly deregulated in human heart failure and significantly correlates with heart failure. Our findings that HypERlnc may have a role in human cardiopulmonary disease. The identification of a HypERlnc orthologue in mouse pericytes will enable to perform translational studies that may substantiate these findings in vivo and elucidate the role of HypERlnc in cardiopulmonary disease.
(Figure 1A), we found that lncRNA HypERlnc was upregulated by hypoxia, which was validated by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Figure 1C demonstrates HypERlnc read coverage under normoxic and hypoxic conditions. Average cycle threshold values for HypERlnc under normoxia were 25.5 and 24.8 under hypoxic conditions, documenting robust expression of the transcript.

Gene expression pattern analyses demonstrate that HypERlnc is expressed in most human organ systems (Online Figure III). Given that not all lncRNAs own polyadenylated tails,15 reverse transcription quantitative polymerase chain reaction in
polyadenylated-enriched RNA fractions was conducted. Here, we found that HypERlnc is polyadenylated (Figure 1D). Because lncRNA function is dependent on subcellular localization, we performed RNA-fluorescent in situ hybridization, demonstrating that HypERlnc is present in the nucleus and in the cytosol of the cell (Figure 1E). Furthermore, reverse transcription quantitative polymerase chain reaction in cytosolic and nuclear fractions indicates that HypERlnc is enriched in the nucleus under both normoxic and hypoxic conditions (Figure 1F).

To evaluate whether HypERlnc is conserved in mice, we performed RNA-seq in primary mouse brain pericytes under normoxic and hypoxic conditions. As the conservation across species is sparse for lncRNAs, we only found 13 commonly annotated lncRNAs in human and mouse pericytes (Online Figure IV A through IVC). However, we found a robust read coverage at the locus that is conserved between the neighboring genes MKL2 and PARN. Murine HypERlnc expression was validated by RT-PCR, suggesting that a murine HypERlnc

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Hypoxia-induced endoplasmic reticulum stress regulating long noncoding RNA (HypERlnc) regulates pericyte differentiation. **A,** HypERlnc knockdown using LNA GapmeRs. **B–E,** Loss of HypERlnc results in cellular dedifferentiation as reflected by a loss of pericyte markers such as platelet-derived growth factor receptor (PDGFR)-β, αSMA, Desmin, and NG2. **F,** RNA-guided gene activation strategy. **G–J,** Pericyte markers PDGFRβ, αSMA, Desmin, and NG2 significantly correlate with HypERlnc expression in gain-of-function experiments. All experiments are n≥3; **P<0.01; ***P<0.001.
orthologue is expressed in mouse pericytes (Online Figure IVD through IVF).

**HypERlnc Knockdown Results in Pericyte Dedifferentiation and Loss of Pericyte Function**

LNA GapmeR-mediated HypERlnc knockdown (Figure 2A) resulted in a downregulation of the pericyte markers platelet-derived growth factor receptor-β, αSMA, Desmin, and NG2 (Figure 2B through 2E), which was confirmed using siRNAs directed against HypERlnc (Online Figure V). Likewise, in HypERlnc gain-of-function experiments using RNA-guided gene activation, the expression of HypERlnc significantly correlated with the expressions of respective pericyte markers, pointing toward an important role of HypERlnc in pericyte differentiation (Figure 2F through 2J). Because pericyte differentiation is important for proper pericyte function, we hypothesized that HypERlnc knockdown impairs the capability of pericytes to induce endothelial barrier function. HypERlnc knockdown in hPC in a coculture model of hPC and human coronary microvascular endothelial cells significantly increased permeability for macromolecules compared with control (Figure 3A and 3B). Next, we studied the impact of HypERlnc knockdown on pericyte recruitment toward endothelial cells because endothelial pericyte recruitment is known to be required for proper endothelial barrier function. HypERlnc silencing significantly reduced hPC recruitment toward human coronary microvascular endothelial cells in Matrigel coculture assays (Figure 3C and 3D). To address whether impairment of pericyte differentiation and recruitment was associated with a loss of viable pericytes or altered pericyte proliferation, we analyzed cell viability and proliferation after HypERlnc knockdown. Here, we found a significant reduction in hPC viability (Figure 3E; Online Figure VIA) and Ki67 staining (Figure 3F and 3G). We did not detect enhanced apoptosis, autophagy, and necrosis by HypERlnc knockdown (Figure 3H; Online Figure VIB through VIE).

**HypERlnc Knockdown Induces Endoplasmic Reticulum Stress**

Because HypERlnc is also present in the nucleus, we tested the hypothesis that HypERlnc regulates transcription factor activity, which is a known molecular mechanism of lncRNAs. Using transcription factor activity luciferase reporter assays in HypERlnc knockdown, we found increased activity of CBF/NF-Y/YY1 and ATF6 (Figure 4A); both of which are known to be involved in the cellular endoplasmic reticulum (ER) stress response pathway. Induction of ER stress was confirmed by enhanced expression of ER stress markers such as IRE1α, soluble BiP, and ATF6 (50 kDa) after HypERlnc knockdown at the protein level (Figure 4B).
Figure 4. Hypoxia-induced endoplasmic reticulum stress regulating long noncoding RNA (HypERlnc) modulates the endoplasmic reticulum (ER) stress response and is regulated in human heart failure. A, Luciferase transcription factor reporter assays in HypERlnc knockdown demonstrate enhanced luciferase activity in CBF/NF-Y/YY1 and ATF6 responsive elements which are part of the ER stress response. B, Representative immunoblots for ER stress markers IRE1α and soluble BiP after HypERlnc knockdown and dithiothreitol (DTT) treatment. DTT was used as a positive control for ER stress. C and D, Quantitative analyses of soluble BiP and IRE1α protein levels confirm enhanced ER stress in LNA HypERlnc-treated pericytes. E, Kyoto Encyclopedia of Genes pathway analyses after RNA sequencing in HypERlnc knockdown demonstrate that vascular smooth muscle cell (VSMC) contraction-related genes and several cardiomyopathies are among the top 20 upregulated pathways. F, HypERlnc expression was analyzed via RT-qPCR in left ventricular myocardium from patients diagnosed with heart failure (HF; n=19). G, HypERlnc RNA levels were significantly reduced compared with controls (n=5 patients without a known history of HF). All experiments are n≥3; *P<0.05; ***P<0.001.
through 4D; Online Figure VII). Interestingly, induction of ER stress significantly lowered HypERlnc levels and induced pericyte dedifferentiation, indicating a regulatory feedback role between the ER stress response and HypERlnc expression that affects pericyte function (Online Figure VII).

**HypERlnc Expression in Cardiopulmonary Disease**

To determine the impact of HypERlnc on gene regulatory pathways, we performed RNA-seq in hPC in HypERlnc knockdown with subsequent analyses of gene ontology (Online Figure VIII and Table V) and Kyoto Encyclopedia of Genes terms. Strikingly, Kyoto Encyclopedia of Genes analysis revealed that genes involved in several cardiovascular disease states and in vascular smooth muscle cell contractility are significantly upregulated (Figure 4E; Online Table VI).

Cardiac disease states, including HF, are known to go along with enhanced ER stress. To address the question whether HypERlnc is regulated in human cardiovascular disease, we measured HypERlnc expression in the left ventricular myocardium of patients diagnosed with HF. HypERlnc was significantly reduced compared with healthy controls (Figure 4F and 4G), corroborating a role of HypERlnc in human cardiovascular disease. We additionally addressed whether HypERlnc is associated with pericyte marker expression in disease states that go along with altered pericyte and vascular smooth muscle cell function such as idiopathic pulmonary arterial hypertension. Although there was no significant difference in HypERlnc levels between healthy donors and idiopathic pulmonary arterial hypertension lungs, we found that HypERlnc significantly correlates with pericyte marker expression in healthy and diseased human lungs (Online Figure IX).

**Discussion**

Here, we characterize the expression and function of HypERlnc in hPCs and demonstrate that HypERlnc is deregulated in human HF and correlates with pericyte marker expression in human lung disease. We show that hypoxia-regulated HypERlnc exerts biologically relevant functions in pericytes by modulating pericyte differentiation, proliferation, and recruitment toward endothelial cells. Mechanistically, loss of HypERlnc resulted in enhanced ER stress. Interestingly, ER stress has been proposed to play a major role in cardiovascular pathology and aging. For example, it has recently been shown that the histone deacetylase sirtuin 1 is cardioprotective by reducing ER stress in cardiac myocytes, thereby inhibiting apoptosis. Moreover, it has been documented that pharmacological inhibition of ER stress in hypertensive mice reduces cardiac injury and results in improved endothelium-dependent relaxation in the aorta. To date, only few lncRNAs have been shown to be associated with the unfolded protein response (UPR), recently demonstrated that Inc-MGC, which is induced by ER stress, is upregulated in a mouse model of diabetic nephropathy. In addition, some studies have shown that IncRNAs influence the UPR. Overexpression of IncRNA MEG3 has been documented to induce ER stress markers (eg, IRE1α) and also induces apoptosis in human hepatoma cells, whereas gain of function of IncRNA TUG1 partly blocked ER stress pathways and was organ protective in a model of cold-induced liver injury in mice. ER stress affects multiple cellular processes and may result in adaptive or proapoptotic pathways. We found a significant upregulation of IRE1α that is known to affect both respective pathways in the UPR and is an ER stress sensor in all eukaryotic cells.

However, we were not able to detect enhanced apoptosis, autophagy, and necrosis in hPCs after HypERlnc knockdown using LNA GapmeRs and siRNAs. hPCs demonstrated cellular dedifferentiation in HypERlnc loss-of-function experiments, which is one of the known physiological effects of UPR.

These results point toward an adaptive UPR response in pericytes after HypERlnc knockdown. This observation is particularly of importance because we silenced HypERlnc using LNA GapmeRs, which may cause apoptosis because of unspecified off-target effects. Because we did not observe enhanced apoptosis in our experimental setting, we conclude that enhanced ER stress is not caused by a nonspecific toxic effect of LNA GapmeRs. In addition, an induction of ER stress resulted in downregulation of HypERlnc and pericyte dedifferentiation, indicating a regulatory feedback loop between ER stress level and HypERlnc expression. We argue that the cellular mechanism for the observed loss of cell viability after HypERlnc knockdown is mediated by a decrease in proliferation. We found that HypERlnc loss significantly downregulates the platelet-derived growth factor receptor-β, which is a tyrosine kinase receptor that is crucial for pericyte proliferation and recruitment.

However, we are aware that the exact mechanism by which HypERlnc loss mediates enhanced ER stress is currently unknown and will need further mechanistic investigation.

With regard to our data on impairment of pericyte proliferation and recruitment toward endothelial cells and impaired coronary endothelial barrier function in HypERlnc knockdown, recent experimental data from myocardial infarction in mice have shown that FOXO4-dependent coronary endothelial barrier breakdown facilitates the migration of inflammatory cells into the myocardial parenchyma, thereby fostering tissue injury. LncRNAs in pericytes may therefore be of clinical relevance in cardiac injury. Moreover, our data on ER stress as well as gene ontology and Kyoto Encyclopedia of Genes analyses in HypERlnc knockdown and reduced expression of HypERlnc in human HF samples corroborate a potential role of HypERlnc in cardiac disease. Furthermore, the clinical relevance of HypERlnc is supported by our findings that HypERlnc significantly correlates with pericyte marker expression in disease states that go along with altered pericyte and vascular smooth muscle cell function such as idiopathic pulmonary arterial hypertension. Further in vivo studies would be desirable to dissect HypERlnc function in disease models such as myocardial infarction or transaortic constriction. However, IncRNA conservation across species is sparse. Particularly the gene loci of mouse IncRNA orthologues lack sequence homology, which makes it difficult to draw a conclusion from mouse data toward human cells and vice versa. Our RNA-seq analyses from mouse pericytes demonstrate a potential mouse orthologue. It will be interesting to develop silencing strategies and study the role of murine HypERlnc in vivo.

In conclusion, our results outline that HypERlnc significantly regulates hPC function and may have a role in human cardiopulmonary disease.
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Disclosures
A.M. Zeiher, C.M. Zehndner, and S. Dimmeler applied for patents related to long noncoding RNAs in pericytes. The other authors report no conflicts.

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Supplemental Material

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Materials and Methods:

Human pericyte cell culture

Human pericytes were acquired from ScienCell and cultured as recommended. Pericytes were used from passages 2-9. Pericytes were cultured in DMEM Glutamax complete with 10% fetal calf serum (both from Gibco, Life Technologies) and Penicillin/Streptomycin (Roche Diagnostics). The humidified atmosphere in the incubator contained 5% CO₂ and 20% O₂ at 37°C. Human coronary microvascular endothelial cells (HCMEC; passages 2-8; ScienCell) were kept in the same atmosphere and cultured in Endothelial Cell Medium (ECM; from ScienCell) containing provided supplements as recommended. For induction of endoplasmic reticulum stress, cells were treated with Dithiothreitol (DTT; 1 mM, 1 hour; Roth) or Tunicamycin (5 µg/ml, 5 hours; Sigma-Aldrich).

Isolation of primary mouse pericytes

Primary mouse brain pericytes were isolated as documented elsewhere from adult C57Bl6 wild type mice with modifications. In brief, mice were euthanized by an overdose of isoflurane followed by decapitation. Brains were quickly removed and transferred into 4°C cold DMEM (Gibco, Life Technologies). Next, olfactory bulb, cerebellum and medulla were dissected and brains were minced with a sterile scalpel. Minced brain tissue was washed once in DMEM, centrifuged for 5 min at 340 g. Medium was carefully discarded and tissue was incubated in an enzymatic digestion mixture in EBSS containing 20 units/ml Papain and 0.005 % DNAse (all from Worthington) for 70 min at 37°C. Following enzymatic digestion, brain tissue was homogenized by passing ten times through an 18 gauge needle and subsequently ten times through a 21 gauge needle. Homogenate was then mixed with 1.7-fold volume of 22% bovine serum albumin in phosphate buffered saline and centrifuged for 10 min at 3800 g. The lipid layer on top was carefully removed and cell pellet was resuspended in collagen coated 6 well plates. During the first three passages, cells were kept in ECM (ScienCell). Starting with the fourth passage, cell culture medium was switched to pericyte medium (ScienCell). Primary mouse pericytes from passage 7-12 were used for experimental manipulation. For validation of murine HypERlnc by reverse transcription PCR, RNA from primary mouse pericytes was isolated using an RNeasy Mini Kit (Qiagen) as recommended including DNA digestion. RNA was reversely transcribed as documented below.

Induction of hypoxia

Cells were kept in pre-equilibrated culture medium in a hypoxic incubator (Labotect) with humidified atmosphere at 5% CO₂, 1% O₂, 37°C. Pericytes were subjected to hypoxia for 24 hours. Hypoxia was verified with measurement of culture medium pO₂ levels with a hypoxia sensing probe (Oxford Optronix) as described elsewhere. Additionally, VEGFA Induction was verified via RT-qPCR and only samples with 2-fold upregulation or higher were used for further analysis.

RNA sequencing in human and murine pericytes

Ribosomal RNA depleted total RNA isolated from human pericytes was analyzed via RNA deep sequencing. The RNeasy Mini Kit (Qiagen) was used to isolate RNA as recommended including DNA digestion. RNA degradation and contamination was monitored on 1% agarose gels and RNA purity determined with a NanoPhotometer® spectrophotometer (IMPLEN). RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies). RNA quality was checked with a RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies). Isolated RNA was fragmented and primed for cDNA synthesis. Libraries were created using a Scriptseq v2 (mouse samples, Illumina, SSV21124) or NEBNext® Ultra™ Directional RNA Library Prep Kit (human samples, Illumina) according to the manufacturer’s instructions. A HiSeq flow cell (Illumina) was used for sequencing. Based on the NONCODE database (noncode.org) IncRNA annotation was performed. RNA-seq of murine pericytes and human pericytes treated with LNA Control or LNA HypERlnc were carried out by Novogene. The uploaded data is accessible on the GEO database when published (with GEO ID: GSE92888).
GO and KEGG enrichment analyses

Gene Ontology (GO) enrichment analysis of differentially expressed genes was done using GOseq R package, in which gene length bias was corrected. GO terms with corrected P value <0.05 were considered significantly enriched by differential expressed genes. KEGG is a database resource for understanding high-level functions and utilities of the biological system, such as the cell, the organism and the ecosystem, from molecular level information, especially large scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies (http://www.genome.jp/kegg/). KOBSAS software was used to test the statistical enrichment of differential expression genes or IncRNA target genes in KEGG pathways.

RNA isolation and reverse transcription quantitative PCR (RT-qPCR)

To isolate total RNA from cell cultures RNeasy Mini Kit (Qiagen) was used as recommended including DNA digestion. Fractionation of RNA for nuclear and cytosolic fractions was performed as described elsewhere5. RNA concentration was measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and transcribed to cDNA using MuLV reverse transcriptase (Life Technologies) and random hexamer primers (Thermo Fisher Scientific) in 40 µl reaction volume. Transcribed cDNA was used with fast SYBR Green (Applied Biosystems) in RT-qPCR performed by a ViiA7 Real-Time PCR System (Thermo Fisher Scientific). CT values were normalized against ribosomal RPLP0 (P0) and relative gene expression was determined through the formula: $2^{-\Delta CT} \quad (\Delta CT = CT_{Target} - CT_{Control})$. The sequences of used primers are given in Online Table III.

Transfection

Cells were transfected after reaching a confluency of 60-80% using Opti-MEM Medium, Lipofectamine RNAiMAX (both from Life Technologies) and 50 nmol/l of LNA GapmeR (Exiqon), siRNA or gRNA blocks. LNA GapmeR control A, scrambled siRNA or transfection medium free of gRNA blocks were used as control for transfection experiments. After 4 hours transfection medium was exchanged to the appropriate cell culture medium. 48 hours upon transfection cells were used for experimental manipulation.

LNA GapmeR sequences were as follows:

LNA HypERlnc: 5’-CTTGGCTGGCGGAAGG-3’
LNA Ctrl: 5’-AACACGTCTATACGC-3’

siRNA sequences directed against HypERlnc were from Sigma-Aldrich ((i)sense: 5’-ACAGCCCUUGUAACUGAU-3’; antisense: 5’-UACAGUUACAAGGGCUGU-3’; (ii) sense: 5’-AGCCCUUGUAACUGAUAC-3’; antisense: 5’-GUUAUCAGUUAACAGGGCU-3’). siRNA controls were transfected with siRNA targeting firefly luciferase as documented elsewhere6 (from Sigma-Aldrich, sense: 5’-CGUACGCAGAUAUUUUGGA-3’; antisense: 5’-UCGAAGUAAUUCGCGGUAC-3’).

RNA guided gene activation

A constitutive dCas9-VP64 lentiviral expression vector carrying a puromycin resistancy (addgene Plasmid #50918, http://www.addgene.org/50918/; kindly provided by Fatma Kok) was used to transduce human pericytes. To verify transduction, pericytes were kept in Pericyte Medium (ScienCell, #1201) containing provided supplements and 1 μg/ml puromycin for at least 6 days to select for successfully transduced pericytes. To achieve overexpression of HypERlnc by RNA guided gene activation, guide RNAs (gRNAs) were designed bearing the antisense sequence to the probable HypERlnc promoter region. gRNAs were designed with the gRNA design toll by the Zhang lab (http://crispr.mit.edu/). gRNA blocks constitutively expressing particular gRNA sequences were bought from IDT and amplified as endorsed by IDT via PCR. Afterwards, pericytes were transfected with the amplification product. Multiple gRNA blocks targeting different promoter region segments were used as described previously7. gRNA block combination and target sequences of gRNAs are shown in Online Table IV. For gRNA block design following sequence scheme was used:
U6 promoter + target sequence (without PAM sequence) + guide RNA scaffold + termination signal

TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAAAAAAAAAAATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTTTTTTTCTAGACCAGCTTTCTTGTAACAAGTGGCATTA

Luciferase reporter array

For analysis of transcription factor activity a Cignal Finder Reporter Array (Qiagen, #336821) was used as recommended by the manufacturer. Human pericytes were transfected with LNA GapmeRs as already described. After transfection, 4x10⁴ pericytes/well were seeded into 96 well plates from Qiagen. Cells were seeded in 50 µl Opti-MEM containing 10% FCS while each well contained 100 µl Opti-MEM supplemented with Lipofectamine RNAiMAX. Cell culture plates were incubated for 4 hours (humidified atmosphere, 5% CO₂, 20% O₂, 37°C). Subsequently, medium was changed to pericyte culture medium and culture plates were incubated for another 24 hours following transfection in the culture plates. Luciferase signals were measured with a GloMax-Multi Detection System from Promega.

Preparation of poly-A⁺ RNA

Preparation was performed as already described⁸. In brief, oligo-d(T)²⁵-magnetic beads (NEB, #S14195) were used to separate poly-A tail positive from poly-A tail negative RNA. Washed beads and RNA were incubated with binding buffer while rotating for 10 min. Separation of poly-A⁺ RNA was done by collecting the supernatant after binding of the beads with a magnet. Afterwards, beads were washed and incubated in elution buffer for 2 min at 50°C to obtain poly-A⁺ RNA. RNA levels were measured with RT-qPCR as described above.

Matrigel co-culture assays

Human pericytes were transduced with a lentivirus for expression of a green fluorescent protein (GFP). Transduction followed standard procedures and precise protocol is available on demand. Transduced pericytes were transfected with LNA GapmeRs as described above. As endothelial cells are known for acetylated LDL uptake⁹, we labeled HCMEC with 10 µg/ml Dil-Acetylated LDL (CellSystems, #CS-D0120) for 16 hours. Matrigel (Corning, #354230) beforehand thawed on ice was transferred in 150 µl doses to pre-cooled 24 or 48 well plates from Corning avoiding bubble formation. Plates were incubated for 30 min (humidified atmosphere, 5% CO₂, 20% O₂, 37°C). 10⁵ stained HCMEC were seeded on top of the matrigel layer in a maximum volume of 500 µl and incubated for 3 hours. Afterwards, medium was removed and 10⁴ GFP-expressing pericytes were added in a maximum volume of 500 µl for 3 hours. Both steps had the same incubation conditions as the matrigel layer. Cell medium was removed and another layer of 150 µl Matrigel was added avoiding bubbles and incubated at described conditions for another 30 min. Pericyte culture medium and ECM (1:1) were added to reach a final volume of 500 µl and the plate was incubated for another 16 hours. Next, cells were fixed for 10 min using 4% Paraformaldehyde (PFA; Roti-Histofix, Carl Roth). 3 randomly chosen fields per view per well were generated using confocal z-stack imaging. GFP-positive pericytes were defined as recruited when attached to HCMEC. Recruited pericytes were counted using Fiji is just ImageJ cell counter tool. The average of recruited pericytes per field per view was calculated.

Barrier function assay

The assay was performed as described previously with modifications¹⁰. In brief, human pericytes were transfected with LNA GapmeRs as described above. ThinCerts (Greiner Bio-One, #662610) with 1 µm pores were coated on the outer pore section with 70 µl of 0.001% Poly-L-Lysin (PLL; Sigma-Aldrich,
#P4832-50ML) in sterile water for 45 min at room temperature. Afterwards, the inner pore section part was coated with 150 µl of 5 µg/cm² fibronectin (FN; Sigma-Aldrich, #F0895-5MG) in PBS (Thermo Fisher Scientific, #14190-094) for 45 min at room temperature. 24 hours after transfection, 3x10⁴ pericytes in 70 µl pericyte culture medium were transferred to the PLL coated outer section for 45 min at room temperature. Subsequently, 3x10⁴ HCMEC in 250 µl ECM were transferred to the FN coated inner section. Cells were incubated for 48 hours (humidified atmosphere, 5% CO₂, 20% O₂, 37°C). 0.25 mg/ml fluorescin Isothiocyanat-dextran (average molecular weight 70,000; Sigma-Aldrich, #FD70S-100MG) in ECM were given in the inner compartment. After 30 min 80 µl out of the outer compartment were transferred to a 96 well plate and fluorescence was measured after excitation with 485 nm using a Synergy HT microplate reader (BioTek).

**Intercellular dye transfer assay and live cell imaging**

HUVEC were seeded on 6 cm dishes. At 60-80% confluency, HUVEC were stained with CellTrace calcein red-orange AM (Life Technologies, #C34851) according to the manufacturer’s instructions. Pericytes were grown to confluency in a culture dish and labeled with CellTrace calcein green AM (5 µmol/l, 30 min; Life Technologies, #C34852). Subsequently, pericytes were washed, trypsinized and transferred into the HUVEC grown culture 6 cm dish at a pericyte/HUVEC ratio of 1:4. After 4 hours of co-culture, live cell imaging was performed using a Zeiss epifluorescent microscope.

**MTT assay**

Human Pericytes were cultured in 24 well plates and transfected with LNA GapmeRs for 48 hours. After removal of culture medium 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid (MTT; 5 mg/ml in PBS; Life Technologies, #M-6494) diluted 1:10 in DMEM (Gibco, #41965-039) was applied. Culture plates were incubated for 1 hour (humidified atmosphere, 5% CO₂, 20% O₂, 37°C). DMEM with MTT was removed and pericytes were resuspended in DMSO. 100 µl of each well were transferred to a 96 well plate and absorption was measured at 565 nm in a Synergy HT microplate reader (BioTek). Blank wells treated with DMEM with MTT and DMSO were used for background subtraction.

**Caspase-3/7 Assay**

Human pericytes (2x10⁴ per well) were seeded in a black 96 well plate (Corning, #353219). Pericytes were transfected as described above. Positive control was treated with 200 nM Staurosporine for 4 hours. All pericytes were stained with 5 µM CellTrace calcein red-orange AM (Life Technologies, #C34851) in pericyte culture medium for 30 min. After washing, cells were incubated with Caspase-3/7 reagent (Apo-One Homogeneous Caspase-3/7 Assay, Promega, #G7790) composited as recommended for 2 hours with light exclusion at room temperature. Fluorescence (calcein red-orange excitation: 577 nm, Caspase-3/7 reagent excitation: 499 nm) was measured with a Synergy HT microplate reader (BioTek).

**Immunofluorescence (IF)**

Standard immunofluorescent staining procedures were done as already described³,⁴,¹¹. In brief, human pericytes were washed with PBS (4°C) and fixed in ice cold acetone (-20°C) for 10 min. Cells were incubated with 7% donkey serum (Dianova) and Triton X-100 in PBS (4°C) for 2 hours at room temperature. Primary antibody incubation (anti-Ki67 and anti-PDGFRβ) was performed with 2% bovine serum albumin (BSA; Dianova), 0.05% azide and 0.1% Triton X-100 in PBS (4°C) for 16 hours at room temperature. Secondary antibodies (anti-rabbit 488 and anti-goat Cy3) and Hoechst (1:1000) were applied in 2% BSA and 0.05% azide in PBS (4°C) for 2 hours at room temperature. Pericytes were embedded in Fluoromount Aqueous Mounting (Sigma-Aldrich, #F4680-25ML) for microscopy.

**Flow cytometry analysis**

For quantification of early apoptotic and necrotic cells, a flow cytometry detection kit was used as recommended by the manufacturer (BD Bioscience, #556547). In brief, human pericytes were grown on 10 cm dishes at a density of 5x10⁵ cells/dish. Transfection procedures were carried out as documented above. For Annexin V labeling, a V450 Annexin V antibody (BD Bioscience, #560506) was used. Cells were starved for 4 hours in Opti-MEM medium in order to induce apoptosis (positive control). For propidium iodide positive controls, cells were treated with 80% ethanol for 5 min at room temperature.
Upon silencing of HypERlnc with siRNA or LNA GapmeRs, cells were washed once with PBS (4°C), trypsinized and centrifuged for 5 min at 1000 g. Washing with PBS and centrifugation was repeated for 2 times. Next, cells were resuspended in binding buffer (4°C). 100 µl of cell suspension (4x10^6 cells/ml) were stained using 5 µl of V450 Annexin V and 5 µl of propidium iodide solution for 15 min at room temperature in the dark. Subsequently, 200 µl binding buffer was added to the vial and measurements were carried out. Analysis was done using a BD FACSCantoTM II flow cytometer and BD FACSDiva Software (from BD Biosciences).

**RNA-fluorescent in situ hybridization (RNA-FISH)**

RNA-FISH probes were designed using the Stellaris probe design tool. Incubation of RNA-FISH oligos directed against HypERlnc labelled with Quasar dye 570 were applied for in situ hybridization as recommended by the manufacturer (Stellaris). MALAT1 RNA-FISH oligos (Quasar dye 570) were used as a positive control. In brief, human pericytes were grown on coverslips. After reaching confluency, cells were exposed towards 24 hours of hypoxia as described above. Following hypoxia, cells were washed with PBS and fixed in 3.7% formaldehyde in RNAse free 1x PBS for 10 min at room temperature. Subsequently, probes were washed twice with PBS and permeabilized using 70% ethanol for at least 1 hour at 4°C. Next, cells were washed as recommended. Finally, probes were hybridized with oligos against HypERlnc or MALAT1 respectively for 4 hours at 37°C. Cells were then washed and counterstained with Hoechst (Invitrogen, 1:2000) for 30 min. After a final washing step cells were embedded in fluoromount and imaging was performed with a Zeiss epifluorescent microscope using an oil objective with 100x magnification.

**Protein isolation, SDS-PAGE, immunoblotting**

Human pericytes were washed with PBS (4°C) and snap frozen in liquid nitrogen. Next, RIPA buffer (4°C; Thermo Fisher Scientific) containing protease inhibitor (Roche Diagnostics) was applied and cells were scraped off with a pre-cooled rubber policeman (-20°C). Pericyte lysate was incubated on ice for 45 min. Next, protein lysate was centrifuged for 10 min with 2350 g at 4°C. Supernatant was carefully removed and transferred to ice-cooled vials. Concentration was measured with a spectrophotometer and Bradford protein assay with Roti-Quant (Carl Roth) following manufacturer’s instructions. Equal volumes of lysate containing 40 µg of protein were mixed with an equal volume of 2x Laemmli buffer (Sigma-Aldrich). Mini-PROTEAN TGX Precast Gels (Bio-Rad) were used for SDS-PAGE at 150 V for 50 min in TBST (Bio-Rad). Gels were blotted using a Pierce G2 Fast Blotter (Thermo Fisher Scientific) as recommended. Western blots (WBs) were blocked with 5% milk/BSA in TBST. Primary antibodies were incubated for 16 hours in 5% milk/BSA in TBST at 4°C. Secondary antibodies were incubated for 1 hour in 5% milk/BSA in TBST at room temperature.

**Induction of autophagy**

In order to block fusion of autophagosome and lysosome cells were treated with 50 µmol/l chloroquine (CQ; Novus Biologicals) for 24 hours or with 100 µmol/l rapamycin (Invivogen) for 4 hours to induce autophagy.

**Confocal microscopy and image analysis**

Fluorescent images were acquired with a Leica SP5 confocal setup (Leica Microsystems). Z-stacks with z-stack step size smaller than 1.8 µm were acquired. Three excitation wavelengths were used (405 nm, 488 nm and 552 nm). Image analysis was done with Fiji is just ImageJ for windows. To analyze Ki67 positive cells, images were randomized. Cells with a positive Ki67 signal were counted. Hoechst stained nuclei were counted after creation of binary images with Fiji automated particle analysis. Ki67 positive cells were set in relation to Hoechst positive cells to determine the relative number of proliferating cells. PDGFRβ was used as a control marker for pericytes.
Patient cohort analyses

Human heart samples

Isolation of left ventricular total RNA was performed with an All-Prep Kit (Qiagen) as recommended including DNA digestion. Left ventricle samples from patients without diagnosis of heart failure (n=5) were compared to samples from patients with diagnosed heart failure (n=19) in RT-qPCR. Participants gave written informed consent to the current study and it was authorized by the ethics committee of the medical faculty of Heidelberg (appl. no. S-390/2011). Symptomatic heart failure patients were consecutively, prospectively enrolled in this study. Control samples were used correctly according to the protected health information (45 C.F.R. 164.514 e2) (Bioserve) and the BCI informed consent F-641-5 (Biochain).

Human lung samples

Human explanted lung tissues from subjects with IPAH (n=7), or control donors (n=7) were obtained during lung transplantation. Samples of donor lung tissue were taken from the lung that was not transplanted. The study protocol for tissue donation was approved by the ethics committee (Ethik Kommission am Fachbereich Humanmedizin der Justus Liebig Universität Giessen) of the University Hospital Giessen (Giessen, Germany) in accordance with national law and with Good Clinical Practice/International Conference on Harmonisation guidelines. Written informed consent was obtained from each individual patient or the patient’s next of kin (AZ 31/93, 10/06, 58/15). All lungs were reviewed for pathology, and the IPAH lungs were classified as grade III or IV according to Heath and Yacoub.

Statistical analyses

Experiments and each experimental condition were carried out n≥3 times if not declared otherwise. Results are shown with mean ± standard error of the mean (SEM). GraphPad 7 for windows (Graphpad) was used for data analysis. Null hypothesis was rejected at α<0.05. The Pearson and D'Agostino omnibus or Shapiro-Wilk normality test was used for normalization control. If passed, the two sided Student’s t-test was used to compare the difference between two groups, if not, analysis was done with the two sided Mann-Whitney U test. Analyses comparing more than two groups were done with One-Way ANOVA with correction for multiple comparisons (Dunnett). Correlation analyses were done using Pearson correlation if data followed Gaussian distribution, or Spearman method if data did not follow a Gaussian distribution. Grubbs’ test was used for outlier detection and outliers were removed for in vitro experiments. Outliers in patient data sets were not removed. Venn diagram was constructed using Venny12.
**A.** FPKM reads of PDGFRβ and NG2 demonstrate robust expression in human primary pericytes. **B.** Immunoblotting confirms expression of PDGFRβ and NG2 in human pericytes.
Online Figure II

Live cell imaging in pericyte-endothelial co-cultures demonstrates formation of functional intercellular junctions between pericytes (green, indicated by asterisks) and HUVEC (red) as indicated by intercellular dye transfer (arrows).
HypERlnc expression in various human organ systems and tissues are displayed. Results for the term “ENSG00000262454” and filtering for Homo sapiens “Organism part” of the EBI Gene Expression Atlas (GXA) are shown:

http://www.ebi.ac.uk/gxa/genes/ENSG00000262454?bs=%7B%22homo%20sapiens%22%3A%5B%20organism_part%5D%7D&ds=%7B%22kingdom%22%3A%5B%22animals%5D%7D#baseline
A. Chart pie diagram of non-coding RNA expression based on RNA-seq analyses in human and B, primary mouse pericytes. C, Venn diagram showing commonly annotated lncRNAs in human and mouse pericytes. D, ECR browser (https://ecrbrowser.dcode.org/) analysis of the human HypERlnc gene position (region highlighted in purple) indicates up to 70% sequence homology (area under the curves in red) in the mouse genome in locus conservation. HypERlnc is flanked by the neighbouring genes MKL2 and PARN. E, RNA-seq in primary mouse pericytes demonstrates high read coverage of the possible mHypERlnc orthologue (in purple). F, Primer alignment and RT-PCR validation of mHypERlnc in primary murine pericytes.
A-C, Targeting HypERlnc with small interfering RNAs results in significant HypERlnc knockdown that goes along with loss of pericyte markers PDGFRβ and NG2. D-F, A combined silencing strategy using both siRNAs and LNA GapmeRs enhances HypERlnc knockdown efficacy that results in de-differentiation of human pericytes. All experiments are n≥3; *P<0.05; **P<0.01; ***P<0.001.
Online Figure VI

A, MTT assay demonstrates significant loss of cell viability upon HypERlnc knockdown using siRNAs and LNA GapmeRs. B, Quantitative analysis of the autophagy marker LC3B II demonstrates that loss of HypERlnc does not induce autophagy. C, Representative protein immunoblotting of LC3B. D, Flow cytometry analysis of annexin V positive cells suggests that loss of HypERlnc does not induce apoptosis. E, Analysis of propidium iodide positive cells suggests that loss of HypERlnc does not induce necrosis. CQ: Chloroquine. All experiments are n≥3; **P<0.01; ***P<0.001

All experiments are n≥3; *P<0.05; **P<0.01
Gene Ontology analysis with regard to biological processes following HypERlnc silencing in RNA-seq demonstrates upregulation of genes affecting cardiovascular system development.
RT-qPCR analyses of HypERlnc and pericyte markers reveal significant correlation of HypERlnc with (A) PDGFRβ, (B) NG2 and (C) Desmin in human lungs derived from healthy donors (n=7) and patients diagnosed with IPAH (n=7).
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## Online Table V

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## Online Table VI

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NF-κappa

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