The Long Noncoding RNA MALAT1 Regulates Endothelial Cell Function and Vessel Growth

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ABSTRACT

Rationale: The human genome harbors a large number of sequences encoding for RNAs that are not translated but control cellular functions by distinct mechanisms. The expression and function of the longer transcripts namely the long non-coding RNAs (lncRNAs) in the vasculature is largely unknown.

Objective: Here, we characterized the expression of lncRNAs in human endothelial cells and elucidated the function of the highly expressed metastasis-associated lung adenocarcinoma transcript 1 (MALAT1; also known as MALAT-1 or NEAT2).

Methods and Results: Endothelial cells of different origin express high levels of the conserved lncRNAs MALAT1, TUG1, MEG3, line00657 and line00493. MALAT1 was significantly increased by hypoxia and controls a phenotypic switch in endothelial cells. Silencing of MALAT1 by siRNAs or GapmeRs induced a pro-migratory response and increased basal sprouting and migration, whereas proliferation of endothelial cells was inhibited. When angiogenesis was further stimulated by VEGF, MALAT1 siRNAs induced discontinuous sprouts indicative of defective proliferation of stalk cells. In vivo studies confirmed that genetic ablation of MALAT1 inhibited proliferation of endothelial cells and reduced neonatal retina vascularization. Pharmacological inhibition of MALAT1 by GapmeRs reduced blood flow recovery and capillary density after hind limb ischemia. Gene expression profiling followed by confirmatory qRT-PCR demonstrated that silencing of MALAT1 impaired the expression of various cell cycle regulators.

Conclusion: Silencing of MALAT1 tips the balance from a proliferative to a migratory endothelial cell phenotype in vitro and its genetic deletion or pharmacological inhibition reduces vascular growth in vivo.

Keywords: lncRNAs, angiogenesis, neovascularization, endothelium, ischemia, non-coding RNA, endothelial function.

Nonstandard Abbreviations and Acronyms:

- IncRNA: long non-coding RNA
- MALAT1: metastasis-associated lung adenocarcinoma transcript 1
- NEAT2: noncoding nuclear-enriched abundant transcript 2
- TUG1: taurine up-regulated gene 1
- MEG3: maternally expressed 3
- siRNA: Small interfering RNA
- VEGF: Vascular endothelial growth factor
- qRT-PCR: Quantitative reverse transcriptase Polymerase Chain Reaction
- ncRNA: non-coding RNA
- miRNA: microRNA
- NATs: natural antisense transcripts
- XIST: X-inactive specific transcript
- Pc2: polycomb 2
- SR: serine/arginine-rich
- HUVEC: Human Umbilical Vein Endothelial Cells
- LNA: locked nucleic acid
- SEM: standard error of the mean
- eNOS: Endothelial nitric oxide synthase
- VEGFR2: vascular endothelial growth factor receptor 2
- PI: Propidium Iodide
- Cyc or CCN: cyclin
- Cdk1: Cyclin-dependent kinase 1
- PCR: Polymerase Chain Reaction
- Rb: retinoblastoma protein
INTRODUCTION

With the use of modern molecular biology techniques like deep sequencing, it has become evident that the majority of the genome is transcribed, while only 2% of the transcribed genome codes for protein. The rest of the transcribed part of the genome is known as non-coding RNA. Non-coding RNAs can be divided in small (<200 nt) ncRNAs, which include microRNAs and transfer RNAs, and longer RNAs (>200 nt) that include ribosomal RNAs and long non-coding RNAs (lncRNAs). Although miRNAs are shown to play important roles in the post-transcriptional regulation of gene expression and to control endothelial cell function, vessel growth and remodeling, little is known about the function of lncRNAs in the endothelium.

Several subclasses of lncRNAs have been described, such as intragenic natural antisense transcripts (NATs) that are transcribed in the opposite direction to a particular gene. LncRNAs can also be located between protein-coding genes or in introns. Moreover, lncRNAs are involved in the regulation of gene expression through epigenetic mechanisms that include chromatin remodeling, the regulation of splicing as well as by acting as sponges for microRNAs. For example, genomic imprinting is controlled by lncRNAs, and the lncRNA XIST is involved in the initiation of X chromosome inactivation. NATs often regulate the associated sense transcript. In endothelial cells, a NAT for tyrosine kinase containing immunoglobulin and epidermal growth factor homology domain-1 (tie-1) was shown to selectively bind tie-1 mRNA and reduced tie-1 transcript levels, resulting in specific defects in endothelial cell contact junctions.

One of the lncRNAs that has been described to control both epigenetic gene regulation as well as splicing is MALAT1, which was first described to be associated with metastasis of lung tumors. MALAT1 was shown to interact with polycomb 2 (Pc2, CBX4) and thereby regulate histone modifications to control cellular proliferation. Furthermore, splicing regulation by MALAT1 was identified in HeLa cells, where MALAT1 interacts with serine/arginine-rich (SR) proteins, thereby regulating subcellular localization of splicing regulating proteins.

Here we characterize the expression of non-coding RNAs in endothelial cells and define a functional role of MALAT1 in the regulation of the angiogenic response of endothelial cells in vitro and vascularization in vivo.

METHODS

Primary cell culture and angiogenesis assays.
Cell culture conditions and methods to determine cell cycle progression, spheroid growth and migration are described in the supplementary method section.

Deep sequencing.
For RNA sequencing, 0.1-4 μg total RNA isolated from HUVEC was used. Poly-A RNA was selected using poly-T oligo-attached magnetic beads. After the elution of the poly-A RNA, the RNA is fragmented and primed for cDNA synthesis. The sequencing libraries were constructed using Illumina® TruSeq™ RNA Sample Preparation Kit, according to the manufacturer’s protocol. Four libraries indexed with different barcodes were pooled and sequenced on one lane of an Illumina HiSeq™ 2000 flowcell. The reads were mapped using tophat with two mismatches allowed. Cuffdiff was used to determine expression of RNA in HUVEC. The lncRNA annotation was based on the NONCODE database (www.noncode.org). The sequence data have been deposited in the NCBI GEO database under accession number GSE54384.
Transfection.
HUVECs were transfected at 60-75% confluence with 100 nmol/L synthesized siRNAs (Sigma, St. Louis, MO) or 1-50 nmol/L LNA GapmeR (Exiqon, Vedbaek, Denmark) targeting MALAT1 using Lipofectamine RNAiMax (Life Technologies, Carlsbad, CA) according to the manufacturer’s protocol. As controls, the siFirefly luciferase or scrambled LNA GapmeR were transfected. Four hours after transfection, the medium was replaced by EBM (Lonza) supplemented with EGM-SingleQuots (Lonza), and 10% FCS (Invitrogen, Carlsbad, CA).

RNA Isolation and Quantitative real-time PCR (qRT-PCR).
Total RNA from cultured cells and mouse tissue was isolated using miRNeasy kits (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Nuclear and cytoplasmic extracts were prepared using the protocol of 13, followed by RNA isolation using miRNeasy Kits (Qiagen). For measuring mRNAs or lncRNAs, 100-1000 ng total RNA was reverse transcribed using MuLV reverse transcriptase (Life Technologies) and random hexamer primers (Thermo scientific, Waltham, MA) in a 20 µl reaction. cDNA was used as template for quantitative real-time RT-PCR (qRT-PCR) using Fast SYBR Green (Applied Biosystems, Forster City, CA) and an Applied Biosystems StepOnePlus machine. Human ribosomal P0 (RPLP0) mRNA was used for normalization. Primer sequences are listed in the supplementary methods. Analysis of relative gene expression levels was performed using the formula $2^{-\Delta\Delta CT}$ with $\Delta\Delta CT = \Delta CT_{\text{target gene}} - \Delta CT_{\text{endogenous control}}$.

Mouse Retinal Angiogenesis Model.
All animal experiments were conducted according to the principles of laboratory animal care as well as according to the German national laws. The studies have been approved by the local ethical committee (Regierungspräsidium Darmstadt, Hessen). MALAT1−/− mice were described previously14. MALAT1+/+ or MALAT1−/− pups were euthanized at post natal day 5. The efficient depletion of MALAT1 in MALAT1−/− mice was validated by qRT-PCR analysis from lung RNA. Retinas were dissected and stained with biotin-labeled isoelectin B4 (1:200) or rabbit anti-phospho-histone H3 antibody (1:100; Millipore), followed by streptavidin secondary antibody or Alexa Fluor–labeled anti-rabbit secondary antibodies (1:400; Invitrogen), respectively, as described previously15. Whole-mount retinas were visualized by confocal microscopy on the laser scanning microscopes LSM780 (Zeiss) and SP2-FCS (Leica) using 10×, 20× and 25×/0.8 (Imm) Plan-Neofluar objectives. Four individual retinal flaps per mouse retina were assessed.

Hind limb ischemia mouse model.
C57bl/6 mice were purchased from Charles River (Sulzfeld, Germany). LNA GapmeR Ctrl (20 mg/kg, Exiqon) or LNA GapmeR MALAT1 (20 mg/kg, Exiqon) were injected intravenously pre-, post-surgery and after 14d. Hind-limb ischemia and subsequent laser-Doppler perfusion measurement and assessment of capillary density were performed as described16.

Microarray experiments and analysis.
Human GeneChip Exon 1.0 ST arrays (“exon arrays”, Affymetrix) were utilized to access the molecular signatures upon the loss of MALAT1. The microarray experiment was performed following the manufacturer’s protocol. CEL files were uploaded to our noncoder web interface 17 and analyzed at the levels of genes and exons. To derive differentially expressed exons, a 2-fold threshold (either inclusion or exclusion of an exon) and $p < 0.05$ were applied to siMALAT1 knockdown compared to the scramble control. The Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources18 were utilized for the analysis of Gene Ontology terms.
Statistical analysis.
Data were analyzed with Graphpad Prism 5 using unpaired student’s t-tests when comparing two conditions, or one-way ANOVA with Bonferroni correction for multiple comparisons. A significance level of p<0.05 was considered significant. Data are presented as mean with error bars depicting the standard error of the mean (SEM).

RESULTS

Characterization of lncRNA expression in endothelial cells.

To determine the expression patterns of non-coding RNAs in endothelial cells, we performed deep sequencing of poly A-selected RNA (Online Table I). This experiment showed that in HUVECs about 56% of the total RNA comprises non-coding RNA (Figure 1A). Of the non-coding RNAs, 7% are annotated as NAT, 7% as long intergenic non-coding RNAs and 42% as other non-coding RNA. Analysis of expression levels identified various lncRNAs that showed expression levels comparable to endothelial coding genes, such as eNOS or VEGFR2 (Figure 1B). The sequences of many lncRNAs are not well-conserved between species, but five of the highest expressed lncRNAs, namely linc00493, MALAT1, MEG3, TUG1 and linc00657, showed considerable sequence conservation between mouse and human (Online Figure I/data not shown).

Therefore, we next focused on these five lncRNAs and confirmed their expression in endothelial cells derived from various human vascular beds by qRT-PCR (Figure 1C). With the exception of MEG3, which was significantly lower in arterial compared to venous or microvascular endothelial cells, all other lncRNAs were expressed at similar levels in the endothelial cells tested (Figure 1C). Since lncRNAs may exhibit different functions depending on their subcellular localization, we additionally determined the levels in nuclear versus cytoplasmic extracts. The known lncRNAs TUG1, MEG3, and MALAT1 were highly enriched in the nuclear fraction, whereas levels of the so far unexplored linc00657 and linc00493 are higher in the cytoplasm (Figure 1D).

To assess whether the expression of the selected lncRNAs is regulated by physiological stimuli, we exposed HUVECs to hypoxia (0.2% O2). These experiments revealed that of the five conserved highly expressed lncRNAs, MEG3 and MALAT1 were profoundly up-regulated by hypoxia with a significant induction at 24h (Figure 1E). Linc00657 and TUG1 were also significantly increased to about 1.5-fold, whereas linc00493 expression was not affected (Figure 1E).

MALAT1 knockdown modulates endothelial cell function in vitro.

Since MALAT1 was highly expressed and most profoundly increased by hypoxia, we further explored the function of MALAT1 in endothelial cells by silencing MALAT1 expression with siRNAs. siRNA treatment resulted in a reduction of total MALAT1 levels compared to scramble controls RNAs (Figure 2A). Importantly, nuclear localized MALAT1 was also significantly down-regulated by siRNA treatment (Figure 2A), despite the observation in a recent report that siRNAs may not target nuclear RNAs19. Silencing of MALAT1 increased basal endothelial cell migration and sprouting as assessed by an in vitro spheroid angiogenesis assay (Figure 2B/C) and scratched wound assay (Figure 2D/E). However, when sprouting was stimulated with VEGF, MALAT1 siRNA treatment did not further increase the outgrowth of spheroids (Figure 2F). Yet, the MALAT1 siRNA treated spheroids showed an interesting phenotype (Figure 2G). Although endothelial cells migrated a similar distance in both groups, the siRNA MALAT1 treated spheroids showed a significantly higher number of discontinued sprouts (Figure 2G/H and online Figure II), indicating that the extension of the sprouts, which is mediated by proliferation of
stalk cells, is disturbed. Indeed, subsequent analysis of endothelial cell proliferation revealed a significant reduction in cell number in MALAT1 silenced endothelial cells (90±4 % compared to control siRNAs after 48 h, p<0.05). The reduced cell number was associated with a significant inhibition of cell cycle progression and MALAT1 silenced reduced the number of cells in S-phase under basal and hypoxic conditions, and after VEGF-stimulation (Figure 2I/J and online Figure III). Cell death as measured by the number of propidium iodide (PI)-positive cells and caspase activity, which is indicative of apoptotic cell death, were slightly, but not significantly, up-regulated in MALAT1 silenced cells (PI+ cells: 125±13%; caspase activity: 151±26 %, compared to control siRNAs).

Since silencing of MALAT1 by siRNA induced an interesting switch from a proliferative to a pro-migratory state of the endothelial cells resulting in an aberrant vessel sprouting under pro-angiogenic conditions, we next aimed to confirm the above observations by using a different approach to silence MALAT1. Therefore, we used LNA™ GapmeRs, which are single-stranded oligonucleotides that consist of a DNA stretch flanked by LNA™ nucleotides. Basepairing with the targeted lncRNA in the nucleus induces degradation by an RNase H-dependent mechanism (Figure 3A). Transfection with GapmeRs directed against MALAT1 at concentrations ranging from 5 – 50 nmol/L silenced human MALAT1 in HUVECs in a dose-dependent manner (Figure 3B). Inhibition of MALAT1 expression by GapmeRs significantly induced angiogenic sprouting of HUVECs under basal conditions, but did not further increase VEGF-stimulated angiogenic sprouting (Figure 3C/D). Moreover, silencing of MALAT1 by GapmeRs for 48h significantly reduced the number of cells (84±4%, p<0.05) and reduced progression through the cell cycle (Figure 3E), thus confirming the results achieved by siRNA-induced silencing of MALAT1 expression.

MALAT1 regulates angiogenesis in vivo.

Since MALAT1 silencing induces a complex switch in endothelial cell function, namely increased migratory and basal sprouting capacity but inhibition of cell cycle progression, we next aimed to address the consequences of these effects for vessel formation in vivo. To this end, we determined vascularization of the neonatal retina at postnatal day 5 in mice lacking MALAT1 expression (MALAT1−/−) (Figure 4A). MALAT1−/− mice showed a delayed vessel extension in the retina compared to wild-type (wt) littermates (Figure 4B/C). Moreover, a reduction of the vessel density, particularly in the front of the vasculature, was observed in MALAT1−/− mice compared to wt littermates (Figure 4B/D). Interestingly, the number of proliferating endothelial cells, as identified by phospho-histone H3 staining, was significantly reduced (Figure 4E/F). However, the number of filopodia was not different in MALAT1−/− compared to wt mice (103±5 %, p=0.95).

To determine whether MALAT1 is required for postnatal neovascularization, we inhibited MALAT1 by GapmeRs in vivo. Intraperitoneal injection of GapmeRs directed against MALAT1 significantly and efficiently suppressed MALAT1 in control and ischemic muscle tissue at day 21 after induction of hind limb ischemia (Figure 4G). Inhibition of MALAT1 significantly inhibited blood flow recovery as determined by laser Doppler imaging and capillary density analysis (Figure 4H/I).

These data confirm the results of the above in vitro studies that silencing of MALAT1 profoundly impairs endothelial cell proliferation, which leads to a block in vessel outgrowth in vitro and in vivo.

MALAT1 modulates the expression of cell cycle regulators.

To gain insights into the mechanism by which MALAT1 regulates endothelial cell function, we performed microarray experiments using RNA of MALAT1 siRNA treated HUVEC, compared to control siRNA treated HUVEC. Bioinformatics pathway analysis revealed that genes involved in cell cycle (p=0.0003) and DNA replication (p=0.021) were most significantly regulated (Online Figure IV). As
illustrated in figure 5A, various critical cell cycle regulatory genes such as cyclins (Cyc or CCN) A2, B1 and B2, and cyclin-dependent kinase 1 (cdk1) were down-regulated by more than 2-fold (Online Table II), whereas the cell cycle inhibitor p21 (CDKN1A) was induced. The control of cell cycle-regulatory genes by MALAT1 was further confirmed by qRT-PCR demonstrating that particularly S-phase cyclins CCNA2, CCNB1 and CCNB2 were significantly down-regulated (Figure 5B). Moreover, the cell cycle inhibitory genes p21 and p27Kip1 were significantly increased upon silencing of MALAT1 (Figure 5B).

As MALAT1 was previously shown to regulate splicing, we next assessed the expression of splicing-related genes, which appeared not to be regulated after MALAT1 silencing (Online Figure V). Furthermore, bioinformatics analysis showed that only few genes in which splicing was significantly regulated and conventional PCR analysis of selected potentially alternatively spliced genes also showed no evidence of alternative splicing (Online Figure VIA/B).

**DISCUSSION**

Here, we demonstrate that many IncRNAs are highly expressed in endothelial cells, including the well-conserved IncRNAs TUG1, MEG3 and MALAT1. We further report a novel, so far unknown function of MALAT1 in endothelial cells and showed that inhibition of MALAT1 induces a switch of the endothelial cell phenotype to a pro-migratory but anti-proliferative state which resulted in impaired endothelial cell proliferation in vitro and in vivo and reduced retinal vessel growth.

The highly expressed IncRNAs MALAT1, TUG1, MEG3 and linc00657 were all further augmented by the exposure of endothelial cells to hypoxia, whereas linc00493 was not. TUG1 regulates retina differentiation and controls the proliferation of tumor cells, but its function in the vasculature is unknown. MEG3 exhibits a tumor suppressive function and MEG3-/- mice show an increased expression of vascular endothelial growth factor A in the brain. Whether the deletion of MEG3 leads to an endothelial cell intrinsic angiogenesis defect is unclear. The function of linc00657 and linc00493 is currently unknown. Although both transcripts are annotated as IncRNAs, they are localized in the cytoplasm, implicating that further studies are mandatory to exclude their function as protein-coding RNAs.

MALAT1 was initially discovered as a tumor-associated IncRNA and was reported to regulate splicing and epigenetic control of gene expression. The function of MALAT1 in the vasculature has not been studied, but the high expression of MALAT1 observed in cultured endothelial cells is consistent with previous results of whole mount in situ hybridization in zebrafish, which showed a strong staining in the vasculature. The data of the present study additionally demonstrates that inhibition of MALAT1 in cultured endothelial cells enhanced sprouting but blocked cell cycle progression in vitro. Although the cell cycle inhibitory activity that we observed after silencing of MALAT1 expression in vitro and in vivo is consistent with previous findings in tumor cells and fibroblasts, the increased sprouting activity was unexpected since previous studies showed that inhibition of MALAT1 in tumor cells severely compromised the motility and migration of tumor cells. In contrast, in the present study, a pro-migratory activity was observed in two different in vitro assays (namely spheroid outgrowth assay and the scratched wound assay) after depleting MALAT1 expression by either siRNAs or GapmeRs. Interestingly, an increase in sprouting after silencing of MALAT1 was only observed under basal conditions whereas no additional increase in sprouting was detectable after VEGF stimulation. This phenotype is likely due to an inhibition of endothelial cell proliferation that prevents the expansion of stalk cells and results in the discontinuation of sprouts. Overall, our data suggest that MALAT1 controls the phenotypic switch from migration to proliferation in endothelial cells, a typical aspect of endothelial cell biology.
The anti-proliferative effect observed after inhibition of MALAT1 is consistent with a profound dysregulation of cell cycle genes observed by gene expression profiling. Previous studies showed that MALAT1 is required for the recruitment of co-activators by Pc2 to the promoters of cell cycle control genes in HeLa cells\textsuperscript{11}, where it serves as a scaffold and physically recruits cell cycle genes into nuclear sub-domains. This is associated with an increase in transcriptionally active histone marks and a decrease in repressive marks. In tumor cells, particularly the expression of CCNE1 and Rb proteins was down-regulated upon MALAT1 depletion. However, in our study, a different set of cell cycle regulators, namely members of the S-phase cyclins A and B, were modulated in endothelial cells. Interestingly, the change in expression of cell cycle regulatory genes in endothelial cells after MALAT1 depletion closely resembled a recently reported gene expression profile of human diploid fibroblasts, which also showed a profound reduction of CCNA2 and CDK1 expression after silencing of MALAT1\textsuperscript{24}. These data indicate that the mechanisms by which MALAT1 controls cell cycle progression may be different in tumor cells compared to somatic cells.

There are conflicting reports about the involvement of MALAT1 in splicing events\textsuperscript{12, 24 25}. In particular, splicing of the oncogenic transcription factor B-myb was proposed to mediate the cell cycle regulatory activity of MALAT1 in fibroblasts\textsuperscript{24}. B-myb expression was also reduced in endothelial cells after silencing MALAT1, but we did not observe a significant regulation of splicing of B-myb (Online Figure VIA). Moreover, we did not observe a transcriptional regulation of splicing factors as shown by others in fibroblasts\textsuperscript{24} (Online Figure V), nor did we observe alternative splicing events in potentially alternatively spliced genes identified in our gene expression profiling (Online Figure VIB). Although we cannot fully exclude that deregulated splicing contributes to the observed effects of MALAT1 in endothelial cells, it appears rather unlikely. These data suggest a cell type or context dependent difference in MALAT1 activity. Further studies are necessary to define the precise molecular function of MALAT1, which may include more than one specific mode of action.

The findings of the present study may have therapeutic implications. MALAT1 is highly expressed in tumor cells\textsuperscript{10} and its inhibition was recently proposed as a novel strategy to block metastasis and tumor growth\textsuperscript{25}. The present study now demonstrates that MALAT1 expression is augmented by hypoxia in endothelial cells and contributes to the proliferative response of endothelial cells. Therefore, one may speculate that the inhibition of MALAT1 may elicit an anti-angiogenic effect in the hypoxic tumor environment that may contribute to a potential therapeutic benefit.

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DISCLOSURES
The authors declare that they have no conflict of interest.
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FIGURE LEGENDS

Figure 1. Evolutionary conserved long non-coding RNAs are highly expressed in endothelial cells and are regulated by hypoxia. A, Polyadenylated RNA of HUVECs was analyzed by deep sequencing. The pie chart indicates different classes of annotated transcripts. B, The 15 highest expressed lncRNAs in HUVECs in comparison to expression levels of eNOS and VEGFR2. Arrows indicate lncRNAs with evolutionary conserved homologues in mouse. C, qRT-PCR analysis of expression levels of the indicated conserved lncRNAs in human endothelial cells derived from umbilical veins (HUVEC), cardiac microvasculature (HCMEC-C), lung microvasculature (HMVEC-L), coronary arteries (HCAEC) and aorta (HAEC). D, HUVEC nuclei and cytoplasm were separated and levels of lncRNAs were measured by qRT-PCR. E, HUVEC were cultured under normoxic or hypoxic (0.2% O2) conditions for 24h. lncRNA expression was measured by qRT-PCR. n≥4, *p<0.05

Figure 2. siRNA-mediated silencing of MALAT1 induces angiogenic sprouting and migration but represses proliferation. A, MALAT1 levels were measured by qRT-PCR in nuclear and cytoplasmic extracts from HUVECs at 48h after transfection with control siRNA (siCtrl) or siRNA directed against MALAT1 (siMALT1). B/C, 48h after transfection with control siRNA or siRNA against MALAT1, spheroids of HUVEC were allowed to sprout in a 3D matrix for 24h. Cumulative sprout length per spheroid was quantified and representative images are shown in panel C. D/E, Scratch wound assay with HUVECs at 48h after transfection with control siRNA or siRNA against MALAT1. Migration was quantified from micrographs at the indicated time points. Representative images are shown in panel E. Cell-covered area is indicated in red. F-H, 48h after transfection with control siRNA or siRNA against MALAT1, spheroids of HUVEC were allowed to sprout in a 3D matrix for 24h in the presence of VEGF121. The distance of migrated endothelial cells was quantified in panel F and representative images are shown in panel G. Arrows indicate discontinued sprouts. The number of discontinued sprouts was quantified in panel H. I/J, Proliferation of HUVECs was measured by flow cytometric BrdU incorporation analysis at 45min after addition of BrdU and 48h after siRNA transfection. All experiments are n≥3, *p<0.05 versus siCtrl.

Figure 3. GapmeR-mediated silencing of MALAT1 induces angiogenic sprouting but represses proliferation. A, GapmeRs are DNA oligonucleotides with locked nucleic acid (LNA) residues at the 3’ and 5’ end which induce RNase H-mediated degradation of nuclear RNA. B, GapmeRs targeting MALAT1 or scrambled control GapmeRs were transfected at increasing concentrations (1-50 nmol/L) and MALAT1 levels were quantified by qRT-PCR after 48h. C/D, GapmeRs were transfected at 50 nmol/L, 48h before spheroids of HUVEC were allowed to sprout in a 3D matrix for 24h. Cumulative sprout length per spheroid was quantified and representative images are shown in panel D. E, Proliferation of HUVECs was measured by flow cytometric BrdU incorporation analysis at 45min after addition of BrdU and 48h after GapmeR transfection. All data are n≥3, *p<0.05

Figure 4. Genetic deletion or inhibition of Malat1 impairs vascularization and proliferation in vivo. A, Malat1 levels in neonatal mouse lungs of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) Malat1 mutant litter mates were measured by qRT-PCR. B-F, In vivo angiogenesis in wild-type and homozygous Malat1 mutant mice was analysed in the mouse neonatal retina model at postnatal day 5. Vasculature was visualized by Isolectin-B4 staining. Outgrowth of the vascular plexus was quantified in C and vessel density in D. E/F, Endothelial proliferation was quantified by phosphorylated histone H3 staining (left panels and shown in red in the merge image). Endothelial cells were stained by Isolectin-B4 (middle panels and green in the merge image) and nuclei in blue (merge image). G/I, Unilateral hind limb ischemia in mice treated with control (white bars) GapmeRs or Malat1 silencing GapmeRs (black bars). Malat1 levels in the ischemic and non-ischemic calf muscles was measured by qRT-PCR (G). Perfusion was measured by laser Doppler imaging (H) and histological capillary density analysis. n≥4, *p<0.05

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Figure 5. MALAT1 regulates expression of cell cycle genes. A, Regulation of cell cycle genes is visualized using exon microarray data of HUVECs transfected with control siRNA or siRNA against Malat1 by superimposing onto the KEGG pathway for cell cycle. Upregulated genes are depicted in red, downregulated in green and low or not expressed genes in white. B, Cell cycle gene expression in HUVECs at 48h after transfection with control siRNA or siRNA directed against MALAT1 was measured by qRT-PCR. n≥3, *p<0.05
Novelty and Significance

What Is Known?

- LncRNAs regulate several cell processes.
- The LncRNA *MALAT1* is highly conserved throughout evolution and expressed in tumor cells. It regulates tumor cell proliferation, apoptosis and migration.

What New Information Does This Article Contribute?

- A comprehensive analysis of long non-coding RNAs present in endothelial cells.
- *MALAT1* is highly expressed in endothelial cells and regulates angiogenic sprouting in vitro.
- Genetic deletion or inhibition of Malat1 impairs vascularization and endothelial proliferation in vivo.

Therapeutic angiogenesis is a promising strategy to augment regeneration of ischemic tissue. lncRNAs have been described to play key roles in cellular processes, but a role for lncRNAs in angiogenesis has not been studied. The lncRNA *MALAT1* is highly expressed in endothelial cells. It is induced by hypoxia and is required for proliferation of endothelial cells. Silencing with GapmeRs or genetic deletion of *MALAT1* reduces neovascularization in mice. Therefore, augmenting *MALAT1* could be a potential strategy to promote therapeutic neovascularization.
Figure 1

A

- mRNA
- Antisense
- lincRNA
- other ncRNAs

B

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C

Rel. expression (% of HUVEC)

Venous Microvascular Arterial

D

Localisation ratio (nuc/cyto)

E

Rel. expression (% of normoxia)

normoxia hypoxia

*
Figure 2

A

Rel. expression of MALAT1 (% of siCtrl)

B

Cum. sprout outgrowth length (% of siCtrl)

C

siCtrl

siMALAT1

D

Migration (μm)

3h 5h

E

Fraction within cell cycle (%)

G0/G1 S G2 + M

F

Cum. sprout outgrowth length (% of siCtrl)

siCtrl siMALAT1

+ VEGF

H

Discontinued sprout length (% of sprout outgrowth length)

siCtrl siMALAT1

+ VEGF

I

Fraction within cell cycle (%) G0/G1 S G2 + M

siCtrl siMALAT1

J

Anti-BrdU

7AAD
Figure 3

Panel A: Schematic representation of LNA™ technology for gapmer (GapmeR) and control (Ctrl) RNA interference. LNA™ molecules are shown to hybridize with complementary DNA strands, leading to RNase H activation and degradation of the targeted RNA.

Panel B: Bar graph showing relative expression of MALAT1 as a percentage of GapmeR Ctrl. Different concentrations of GapmeR (50nM, 1nM, 5nM, 10nM, 50nM) are tested against MALAT1 expression levels.

Panel C: Cumulative sprout length as a percentage of GapmeR Ctrl. Comparison between GapmeR Ctrl and MALAT1 in the presence and absence of VEGF.

Panel D: Micrographs of cell morphology showing the effect of VEGF on GapmeR Ctrl and MALAT1.

Panel E: Fraction within cell cycle (% G0/G1, S, G2 + M) for GapmeR Ctrl and MALAT1 with and without VEGF.
Figure 4

A  Relative Malat1 levels (% of Malat1+/+)

B  Outgrowth (% of Malat1+/+)

C  Phospho histone H3 (endothelial cells/mm²)

D  Vessel density (% area)

E  Phospho histone H3 (endothelial cells/mm²)

F  p-H3 IB4 Merge

G  Relative expression MALAT1(%)

H  Laser Doppler-derived blood flow (% of GapmeR Ctrl)

I  Capillaries/mm²
The Long Noncoding RNA MALAT1 Regulates Endothelial Cell Function and Vessel Growth

Katharina M Michalik, Xintian You, Yosif Manavski, Anuradha Doddaballapur, Martin Zörnig, Thomas Braun, David John, Yuliya Ponomareva, Wei Chen, Shizuka Uchida, Reinier A Boon and Stefanie Dimmeler

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Supplemental Material

Supplementary methods

Primary Cell Culture Conditions

Human umbilical vein endothelial cells (HUVEC), human lung microvascular endothelial cells (HMVEC-L), human coronary artery endothelial cells (HCAEC) and human aortic endothelial cells (HAEC) were purchased from Lonza (Verviers, Belgium) and cultured in endothelial basal medium (EBM; Lonza) supplemented with EGM-SingleQuots (Lonza), and 10% fetal calf serum (FCS; Invitrogen (San Diego, CA) until the third passage. Human cardiac microvascular endothelial cells (HCMEC-C) were purchased from PromoCell and cultured in endothelial cell growth medium MV kit (PromoCell, Heidelberg, Germany) until the third passage. Cells were cultured at 37°C and 5% CO2 and the cell number was determined by NucleoCounter (ChemoMetec A/S, Allerød, Denmark).

Cell Cycle Analysis

For quantification of cell proliferation, transfected HUVEC were incubated with BrdU (10 mmol/L) for 45 minutes. Adherent cells were detached with trypsin, washed in PBS and incubated with 2.5 μL anti-BrdU-V450 in PermWash buffer (BD Biosciences Franklin Lakes, NJ) for 20 minutes at RT and with 20 μL 7AAD for 10 minutes according to the manufacturer (BD Pharmingen, BrdU Flow Kit). Analysis was performed using a BD FACSCantoTM II flow cytometer and BD FACSDiva Software (BD Biosciences).

Caspase activity assay

Caspase activity assay was performed according to the manufacturer’s protocol for Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Madison, WI). Fluorescence was measured with a Glomax Multi plate reader (Promega).

Spheroid Assay

Cell spheroids of HUVECs were generated as described previously. In vitro angiogenesis was quantified by measuring the cumulative length of all sprouts of each spheroid or the maximal distance of the migrated cells (“sprout outgrowth length”) using digital imaging analysis software (AxioVision Rel. 4.8, Carl Zeiss). 10-12 spheroids were analyzed for each experiment.
Migration

12-well culture plates were coated with 1 μg/mL human fibronectin (Sigma Aldrich) overnight at RT. Then, culture-inserts (Ibidi) were placed on the bottom of the wells. Transfected HUVEC were detached with trypsin after 24h and plated in the wells with the culture-inserts and incubated overnight at 37°C and under 5% CO2. Next day, the culture-inserts were removed, and the cell monolayer was rinsed once with PBS. Micrographs were taken from the wounds at time points 0h, 3h and 5h using a 5x objective on a Zeiss Observer Z1 microscope. The mean migration distance was quantified using AxioVision 4.8 software (Carl Zeiss MicroImaging).

Primer and siRNA sequences

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<th>Sequence</th>
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TaqMan® Array Human Cyclins & Cell Cycle Regulation (Cat.No 4414123)
<table>
<thead>
<tr>
<th>siRNA/LNA GapmerR</th>
<th>Sequence</th>
</tr>
</thead>
</table>
| siFirefly luciferase (siCtrl) | CGUACGCGGAUACUUCGAdTdT (sense)  
                          | UCGAAGUAAUGCGUACG (antisense) |
| siMALAT1                | GUAAAGCCCGACUACUUCGAdTdT (sense)  
                          | UGAUAGUUCAGGGCUUUACdTdT (antisense) |
| GapmeR Ctrl             | GACTAATGCATTATC                                                 |
| GapmeR MALAT1           | GTCACAATGCATTCTA                                                 |
Online figure I. MALAT1 is evolutionarily conserved.
The top graph shows in red the read coverage of deep sequencing analysis at the Malat1 locus in HUVECs. The annotated human Malat1 transcript is shown in blue (top graph) or yellow (bottom graph). The bottom graph shows the evolutionarily conservation of Malat1 in different species (in yellow peaks), compared to the human genome (adapted from rVISTA 2.0, http://rvista.dcode.org/).
Online figure II. Silencing of MALAT1 increases discontinued sprouts after VEGF treatment.

48h after transfection with control siRNA or siRNA against MALAT1, spheroids of HUVEC were allowed to sprout in a 3D matrix for 24h in the presence of VEGF121. Discontinued sprouts (indicated by arrows), as quantified in figure 2H, were determined by measuring cumulative sprout length and cumulative sprout outgrowth length (see cartoon on the left). Scale bar indicates 100 µm.
Online figure III. Silencing MALAT1 reduces proliferation, but only slightly induces apoptosis.

(A) Cell cycle progression (BrdU assay) or (B) apoptosis (caspase activity assay) was analyzed in SiControl and siMALAT1 transfected HUVEC under normal cell culture conditions, after hypoxia treatment or after stimulation with VEGF. The number of cells in S-phase is depicted as percentage of total cell number in panel A. Staurosporine in panel B is used a positive control for apoptosis induction. *p<0.05. n=3 (A) and n=6 (B).
Online Figure IV

<table>
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<th>REACTOME Pathway</th>
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<th>p-value</th>
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<td>DNA Replication</td>
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<td>2.1</td>
<td>0.0210</td>
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<tr>
<td>Cell Cycle Checkpoints</td>
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<td>DNA Repair</td>
<td>8</td>
<td>1.5</td>
<td>0.3400</td>
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<tr>
<td>Signaling by Rho GTPases</td>
<td>8</td>
<td>1.5</td>
<td>0.5500</td>
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<tr>
<td>Telomere Maintenance</td>
<td>6</td>
<td>1.1</td>
<td>0.3200</td>
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</tbody>
</table>

**Online figure IV. Silencing MALAT1 affects the expression of cell cycle regulating genes.**

SiControl and siMALAT1 transfected HUVEC were used for microarray analysis. **A**, Genes that were significantly (p<0.05) up- or downregulated (>2-fold and <0.5 fold, resp.) after MALAT1 silencing were used for Reactome pathway analysis (http://david.abcc.ncifcrf.gov). “Count” indicates the number of regulated genes that are present in the pathway and “%” indicates the relative number of regulated genes in the pathway compared to the total number of genes in the pathway. P-values according to Benjamini-Hochberg statistics (FDR) are depicted. **B**, M/A plot showing genes detected by microarray analysis and highlighted in blue are cyclins, cyclin dependent kinases or other cell cycle regulators.
Online Figure V. MALAT1 silencing does not affect splicing gene expression.

Heatmap showing microarray data of HUVEC treated with siControl or siMALAT1 (this study) compared to microarray data of fibroblasts after silencing MALAT1 or control conditions (Tripathi et al). Only genes shown by Tripathi and colleagues to be involved in splicing and regulated by MALAT1 silencing are depicted. Clustering was performed with this subset of genes. Yellow depicts upregulation and blue downregulation after silencing MALAT1.
Online figure VI. B-Myb, NUF2, PRIM2 and CTD1 are not alternatively spliced after MALAT1 silencing in HUVECs.

A, Boxplot showing microarray data of the probes for B-Myb in HUVECs treated with siControl (red) or siMALAT1 (blue) (this study) and fibroblasts after silencing MALAT1 (green) or control conditions (black) (Tripathi et al). B, NUF2, PRIM2 and CTD1 showed potential alternative exon use after siMALAT1 by microarray. PCR confirmed that in all cases only one splice variant is expressed.
## Online Table I

<table>
<thead>
<tr>
<th>Associated Gene Name</th>
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<th>HUVEC 1</th>
<th>HUVEC 2</th>
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<tr>
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<td></td>
<td>37190680</td>
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<td># mapped (%)</td>
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<td>35962472 (96.70)</td>
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<td># within gene (%)</td>
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<td>35048355 (94.24)</td>
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<td>TOP 15 lncRNAs</td>
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<td>23.8640654</td>
<td>27.2374918</td>
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</table>

**Online table I. Number of reads per HUVEC sample.**

(Top part) Polyadenylated RNA of two independent HUVEC cell lysates was sequenced using an Illumina Hiseq™ 2000 flowcell. The total number of reads per sample, the number of reads that were mapped to the human genome using tophat and the number of reads that were mapped to annotated genes are depicted. Percentages indicate % of raw reads. (Bottom part) Expression levels (RPKM) of the 15 highest expressed annotated lncRNAs are depicted for both biological HUVEC replicates.
Online Table II

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<tr>
<th>Cluster ID</th>
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</table>

Online table II. Silencing MALAT1 affects the expression of cell cycle regulating genes. Microarray analysis of HUVECs after siMALAT1 or siControl transfection.