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Original article

Long noncoding RNA LISPR1 is required for S1P signaling and endothelial cell function

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ABSTRACT

Sphingosine-1-Phosphate (S1P) is a potent signaling lipid. The effects of S1P are mediated by the five S1P receptors (S1PR). In the endothelium S1PR1 is the predominant receptor and thus S1PR1 abundance limits S1P signaling. Recently, lncRNAs were identified as a novel class of molecules regulating gene expression. Interestingly, the lncRNA NONHSAT004848 (LISPR1, Long intergenic noncoding RNA antisense to S1PR1), is closely positioned to the S1P1 receptors gene and in part shares its promoter region. We hypothesize that LISPR1 controls endothelial S1PR1 expression and thus S1P-induced signaling in endothelial cells. In vitro transcription and translation as well as coding potential assessment showed that LISPR1 is indeed noncoding. LISPR1 was localized in both cytoplasm and nucleus and harbored a PolyA tail at the 3'end. In human umbilical vein endothelial cells, as well as human lung tissue, qRT-PCR and RNA-Seq revealed high expression of LISPR1. S1PR1 and LISPR1 were downregulated in human pulmonary diseases such as COPD. LISPR1 but also S1PR1 were induced by inflammation, shear stress and statins. Knockdown of LISPR1 attenuated endothelial S1P-induced migration and spheroid outgrowth of endothelial cells. LISPR1 knockdown decreased S1PR1 expression, which was paralleled by an increase of the binding of the transcriptional repressor ZNF354C to the S1PR1 promoter and a reduction of the recruitment of RNA Polymerase II to the S1PR1 5'end. This resulted in attenuated S1PR1 expression and attenuated S1P downstream signaling. Collectively, the disease relevant lncRNA LISPR1 acts as a novel regulatory unit important for S1PR1 expression and endothelial cell function.

1. Introduction

S1P1 receptor (Sphingosine-1-Phosphate Receptor 1) is a G-protein coupled receptor and one of five receptors with high affinity for S1P [1]. Through the S1P1 receptor, S1P modulates several pathways in a broad spectrum of cell types [2,3]. In endothelial cells, S1PR1 is highly expressed and involved in ERK/MAPK signaling in response to shear stress [4,5]. The transcription factor Krüppel-like factor 2 has been shown to be involved in S1PR1 transcriptional regulation [6]. In the vascular system, S1P promotes adherens junction assembly through the GTPases Rho and Rac and potentiates FGF-2- and VEGF-induced angiogenesis and sprouting of human umbilical vein endothelial cells [7,8]. Moreover, S1P stimulates proliferation and migration of endothelial cells [9] and maintains endothelial cell barrier integrity [10]. S1P potentiates agonist-stimulated sprouting of HUVECs [8] and S1P1 receptor knockout mice die early in embryogenesis due to incomplete vascular maturation [11]. This is in part consequence of an insufficient vascular coverage with vascular smooth muscle cells, an effect directed

Abbreviations: COPD, Chronic obstructive pulmonary disease; CTEPH, Chronic thromboembolic pulmonary hypertension; CRISPR/Cas9, Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9; HUVEC, Human umbilical vein endothelial cells; IPAH, Idiopathic pulmonary arterial hypertension; LISPR1, Long intergenic noncoding RNA antisense to S1PR1; LncRNA, Long noncoding RNA; MZF1, Myeloid Zinc Finger 1; S1P, Sphingosine-1-phosphate; S1P1, Sphingosine-1-Phosphate Receptor 1 protein; S1PR1, Sphingosine-1-Phosphate Receptor 1 gene; ZNF354C, Zinc Finger Protein 354C

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by endothelial S1P1 receptor [12]. Laminar shear stress and circulating S1P activate endothelial S1P1 receptor and stabilize the vessel [5].

The role of epigenetic mechanisms and the influences of lncRNAs in the context of S1P are unknown so far. LncRNAs are defined as transcribed, but not translated RNA molecules > 200 nucleotides in length [13–15]. These lncRNAs have become central in exerting regulatory roles in nearly all cellular processes. Many lncRNAs control gene expression either on the level of epigenetics or transcription [16], e. g. lncRNA Khps1, which forms a DNA:DNA:RNA triplex upstream of the sphingosine kinase 1 transcriptional start site and recruits the histone acetyltransferase p300/CBP for local chromatin changes, thereby ensuring E2F1 binding and enhancing transcription [17]. LncRNAs are also known to regulate their gene neighborhood in cis [18]. Moreover, promoter associated lncRNAs have been identified with gene and protein regulatory potential [19,20]. An importance in the vascular system has been reported for some lncRNAs e. g. MALAT1 inhibits neonatal retina vascularization [21] and FENDRR, BRAVEHEART, ALIEN, TERMINATOR and PUNISHER are involved in heart development [22–24]. In contrast to this, the functions and mechanisms of the majority of promoter associated lncRNAs are unknown. Since lncRNA-dependent vascular disease analyses are just in their beginnings, only a handful of lncRNAs have been found to be involved in these processes. Among them are SENCR (smooth muscle cell function [25]), MANTIS (pulmonary hypertension, glioblastoma and global endothelial cell function [26]), NONHSAT073641 (Chronic thromboembolic pulmonary hypertension [27]), HOTAIR (aortic valve calcification [28]), MHRT (heart hypertrophy [29]), CHRF (Cardiac hypertrophy [30]) and CARL (myocardial infarction [31]).

Here, we analyzed the capability of the lncRNAs involved in S1P signaling. We identified the lncRNA LISPR1 which maintained S1PR1 gene expression. LISPR1 expression was decreased in pulmonary vascular disease and knockdown of LISPR1 decreased S1PR1 expression. Functionally, this resulted in attenuated S1P1 receptor-dependent signaling of S1P.

2. Material and methods

2.1. Cell culture

Pooled human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (CC-2519, Lot No. 371074, 369146, 314457, 192485, 186864, 171772, Walkersville, MD, USA) and PELOBiotech (PB-CH-190-813, Lot No. QC-18P13F11, Planegg, Germany). Immortalized human microvascular endothelial cells (HMEC) (#98247) were acquired from the CDC (Atlanta, GA, USA) and Human aortic endothelial cells (HAoEC) were purchased from PeloBiotech (304 K-05a). HUVECs, HAoECs and HMECs were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. To culture the cells, Fibronectin-coated (356009, Corning Incorporated, USA) dishes were used. Endothelial growth medium (EGM), consisting of endothelial basal medium (EBM) supplemented with human recombinant epidermal growth factor (EGF), EndoCGS-Heparin (PeloBiotech, Germany), 8% fetal calf serum (FCS) (S0113, Biochrom, Germany), penicillin (50 U/mL) and streptomycin (50 µg/mL) (15140-122, Gibco/Lifetechnologies, USA) was used. For HAoEC, 16% FCS was used instead of 8%. For each experiment, at least three different batches of HUVEC from passage 3 were used.

Human aortic smooth muscle cells (HAoSMC, 354-05a), human coronary artery smooth muscle cells (CASMC, 350-05a) and human carotid artery smooth muscle cells (CTASMC, 3514-05a) were purchased from PeloBiotech (Planegg, Germany) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C in smooth muscle cell medium (PB-MH-200-2190) supplemented with 8% FCS, penicillin (50 U/mL), streptomycin (50 µg/mL), EGF, fibroblast growth factor, glutamine, and insulin from singlequots (PeloBiotech, Planegg, Germany).

Human dermal lymphatic endothelial cells (HDLEC, #3111203.4, #394Z027.3, #4092401.3) were purchased from Promocell (Heidelberg, Germany) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C in endothelial cell growth medium MV2 (Promocell, Heidelberg, Germany).

Human embryonic kidney (HEK) 293 cells were obtained from ATCC (Manassas, VA, USA) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C in Dulbecco's Modified Eagle Medium (DMEM), high glucose, GlutaMAX from Gibco, Lifetechnologies (Carlsbad, CA, USA), supplemented with 8% FCS, penicillin (50 U/mL), and streptomycin (50 µg/mL).

Human foreskin fibroblasts were purchased from Gibco (Lifetechnologies, Carlsbad, CA, USA) and cultured in DMEM/F12 (#11039-021) supplemented with 10% FCS, penicillin (50 U/mL), and streptomycin (50 µg/mL) in a humidified atmosphere of 5% CO_2 at 37 °C.

The human monocytic cell line THP-1 and the cancer cell line MCF-7 were obtained from ATCC (LGC Promochem, Wesel, Germany) and cultured in RPMI medium containing stable glutamine, 8% FCS and 1% penicillin/streptomycin (PAA Laboratories, Cölbe, Germany) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.2. Materials and stimulation

The following chemicals and concentrations were used for stimulation in cell culture experiments: Human recombinant VEGF-A 165 (293-VE, R&D, 50 ng/mL), S1P (26993-30-6, Cayman Chemical, 30 nM, 100 nM, 1 µM), human recombinant TNF-α (300-01A, Peprotech, 10 ng/mL), Cerivastatin (143201–11-0, Cayman Chemical, 50 nM), Fluvastatin (Novartis, 100 nM), Atorvastatin (LKT-A7658, Biomol, 100 nM) and Simvastatin (10010345, Cayman Chemical, 150 nM).

2.3. Human lung samples and patient characteristics

Human explanted lung tissues from subjects with Chronic obstructive pulmonary disease (COPD, $n = 8$), chronic thromboembolic pulmonary hypertension (CTEPH, n = 9), idiopathic pulmonary artery hypertension (IPAH, n = 8) or control donors (n = 9) were obtained during lung transplantation. Samples of donor lung tissue were taken from lungs that had not been transplanted. All lungs were reviewed for pathology. The ethics committee of the University Hospital Giessen (Giessen, Germany) approved the study protocols. 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).

2.4. RNA isolation, reverse transcription and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated with the RNA Mini Kit (Bio&Sell). For reverse transcription, SuperScript III Reverse Transcriptase (Thermo Fisher) and oligo$(dT)_{23}$ together with random hexamer primers (Sigma) were used. Copy DNA amplification was measured with qRT-PCR in a MX3005 qPCR system (Stratagene). ITaq Universal SYBR Green Supermix and ROX as reference dye (Bio−Rad, 1725125) were used. Relative expression of target genes was normalized to β-Actin and analyzed by the delta-delta Ct method with the Mx qPCR software (Stratagene). Oligonucleotides used for amplification are listed in Table 1.

For expression analysis of LISPR1 and S1PR1 in 20 different human tissues, 1 µg of RNA from the Human Total RNA Master Panel II (636643, Clontech, Mountain View, CA, USA) was reverse transcribed.