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Cerebrovascular Angiogenic Reprogramming upon LRP1 Repression: Impact on Sphingosine-1-Phosphate-Mediated Signaling in Brain Endothelial Cell Chemotactism

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Abstract Switches in sphingolipid metabolism have recently been associated with oncogenic transformation, and a role for the low-density lipoprotein receptor-related protein 1 (LRP1) in sphingosine-1-phosphate (S1P) proangiogenic signaling inferred. S1P signaling crosstalk with LRP1 in brain microvascular endothelial cells (HBMEC) is however unclear. Transient in vitro siLRP1 gene silencing was compared to stable shLRP1 knockdown. We observed decreased expression of CCAAT/enhancer binding protein β (C/EBPβ), a transcription factor for which multiple binding sites are found within the promoter sequences of all five S1P receptor members, upon stable but not transient LRP1 repression. Chemotactic migration of brain EC isolated from *Lrp1*(EC)$^{-/-}$ mice and of stable shLRP1 HBMEC became unresponsive to S1P, partly due to altered ERK and p38 MAPK pathways, whereas chemotactism remained unaltered following transient in vitro siLRP1 repression. Diminished *S1P1*, *S1P3*, and *S1P5* expression were observed in stable shLRP1 HBMEC and in brain EC isolated from *Lrp1*(EC)$^{-/-}$ mice. Overexpression of LRP1 cluster IV rescued S1P-mediated cell migration through increased *S1P3* transcription in shLRP1 HBMEC. Our study highlights an adaptive signaling crosstalk between LRP1 and specific S1P receptors which may regulate the angiogenic response of brain EC and be targeted at the blood-brain barrier in future therapeutic strategies.

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Keywords Blood-brain barrier · Brain endothelial cells · LRP1 · Sphingosine-1-phosphate · Angiogenesis

Introduction

Glioblastomas (GBM) are highly angiogenic tumors where important metabolic reprogramming enables them to efficiently use altered metabolic enzymes and their oncogenic metabolites [1]. Reprogramming of metabolism in cancer cells includes changes in the expression of genes that directly control the rate of key metabolic pathways such as glycolysis, lipogenesis, and nucleotide synthesis, which are dysregulated upon the adaptation and progression of tumor cells towards more aggressive phenotypes [2]. While metabolic shifts, such as the Warburg effect of enhanced aerobic glycolysis, are relatively well-characterized in the cancer cell compartment [3], our knowledge concerning the adaptive mechanisms taking place at the blood-brain barrier (BBB) and specifically within the cerebrovascular endothelial cell (EC) compartment remains extremely limited.

Among the growth factor- and cytokine-mediated angiogenic cues released from brain cancer cells and which affect the BBB and the tumor microenvironment, sphingosine-1-phosphate (S1P) is a pleiotropic lipid mediator regulating cell survival, migration, recruitment of immune cells, angiogenesis, and lymphangiogenesis, all processes involved in cancer progression [4]. A shift towards increased S1P synthesis is, in fact, observed in GBM [5], and results in tumor cell survival and resistance to chemotherapy. High expression of sphingosine kinase (SphK1/2), the enzyme responsible for transforming sphingosine into S1P, was proven to correlate with poor survival for patients with GBM [6]. Furthermore, the use of maintenance therapy with a SphK inhibitor, in

patients with GBM who had tumor reduction or stable disease after therapy, has been investigated [7–10].

Metabolic reprogramming is also observed in response to increased cell proliferation and growth and during high turnover of cell cholesterol for membrane growth [11]. Cells requiring cholesterol for membrane synthesis may take up plasma LDL-3, the main cholesterol carrier in blood, via receptor-mediated endocytosis [12]. This form of endocytosis is thought to involve elevated LDL receptor levels in rapidly growing tumor cells [13]. Consequently, high expression of LDL receptor family members on numerous glioblastoma cells could potentially be useful for targeting of antitumor agents [14]. Interestingly, LRP1 has been reported to serve as a receptor-mediated transcytosis intermediate for drug delivery across the BBB by binding to the targeting ligand Angiopep-2 [15, 16]. Recently, switches in sphingolipid metabolism have been associated with oncogenic transformation, and a role for LRP1 in S1P proangiogenic signaling has been inferred. The functional relevance of S1P/LRP1 signaling crosstalk remains, however, to be investigated. Moreover, LRP1 was shown to interact with the sphingolipid signaling complex and to promote development of vascular smooth muscle cells [17]. The molecular basis of LRP1's interaction with the sphingolipid signaling complex is poorly understood in the context of brain tumor-driven angiogenesis.

The purpose of this study was to examine whether any adaptive S1P/LRP1/S1P receptor interdependence mechanism was required in the regulation of brain EC migration. We compared cell chemotactism in response to S1P in cells where *Lrp1* had been transiently silenced to a stable shRNA-mediated LRP1 knockdown in a human brain microvascular EC model. We also analyzed the adaptive transcriptional crosstalk between LRP1 and specific S1P receptors in murine brain EC isolated from *Lrp1*(EC)$^{-/-}$ mice. Our current study will enable us to highlight any adaptive transcriptional crosstalk linking LRP1 to specific S1P receptors which would account for S1P signaling in brain EC. A better understanding of the S1P/LRP1 signaling axis at the BBB may ultimately lead to the design of targeted anti-angiogenic strategies.

Experimental Procedures

Materials Sodium dodecylsulfate (SDS), S1P, lysophosphatidic acid (LPA), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Canada (Oakville, ON). Electrophoresis reagents were from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Perkin Elmer (Waltham, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The antibodies against phospho-ERK1/2 (4377), phospho-JNK (9251), phospho-p38 MAPK (9211), ERK1/2 (9107), JNK (9252), and p38 MAPK (9212) were all purchased from Cell Signaling Technology (Danvers, MA).

Cell Culture Human brain microvascular endothelial cells (HBMEC) were immortalized by transfection with simian virus 40 large T antigen, a model that closely mimics the brain tumor endothelium phenotype, and maintained their morphological and functional characteristics for at least 30 passages [18]. HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS, HyClone Laboratories, Logan, UT), 10% (*v*/v) NuSerum (BD Bioscience, Mountain View, CA), and EC growth supplement (30 μg/mL). Human *Lrp1* gene silencing within HBMEC was performed using the pcDNATM6.2-GW/EmGFP-miR vector system where a specific human *Lrp1*-shRNA was cloned (Hmi409872_top_LRP1 and Hmi409872_bot_LRP1; Invitrogen). Cells were transfected with either this construct or with the empty vector using Lipofectamine reagent (Invitrogen) and selected 48 h later with 6 μg/mL Blasticidin. Resistant clones were isolated and the clone which exhibited the best silencing of LRP1 expression was validated by Western blot and qPCR. GFP-HBMEC and LRP1$^{-/-}$ (GFP, clone 4.3)-HBMEC were maintained in RPMI 1640 supplemented with 10% (*v*/*v*) FBS, 10% (*v*/*v*) NuSerum, EC growth supplement (30 μg/mL), and blasticidin S (12 μg/ml). Cells were cultured at 37 °C under a humidified atmosphere containing 5% CO_2.

Mouse Brain Endothelial Cell Isolation All animal experiments were evaluated and approved by the Institutional Committee for Good Animal Practices (UQAM, Montreal, QC). Genotyped 6–8-week-old C57/BL6 *Lrp1*(EC)$^{-/-}$ mice were obtained through breeding of LRP1$^{flox/flox}$ (B6;129S7-Lrp1^{tm2Her}/J) and Tie2-Cre (B6.Cg-Tg(Tek-cre)^{12Flv}/J) animals in which Cre recombinase expression is driven by a mouse endothelial-specific receptor tyrosine kinase promoter/enhancer. Recombinase activity results in the deletion of loxP flanked targets in the female germline as well as in endothelial cells (Jackson Laboratories, Bar Harbor, MA). The mouse brain EC isolation procedure was adapted from previous studies [19–21]. WT and *Lrp1*(EC)$^{-/-}$ mice were euthanized under CO_2. Brains of 4 to 10 mice were removed, taking care to detach the cerebellum, meninges and large vessels. The brains were rinsed in DMEM containing 2% FBS then cut into small pieces of 1–2 mm^3 and placed in tubes containing DMEM-FBS. The brains were homogenized manually using a Potter-Elvehjem tissue grinder, and homogenates mixed with an equal volume of 31% (*w*/*v*) Dextran 70 and stirred for 20 min at 4 °C. The mixes were centrifuged at 10,000*g* for 20 min at 4 °C. The thick, white supernatant (myelin) was removed. The pellet was resuspended in a 0.05% collagenase A solution (in PBS) before being incubated for 1 h at 37 °C. The solutions were then filtered through a 180 μm Nitex filter

and then filtered through a 30 μm Nitex filter. The filtrates were centrifuged at 600 g for 10 min at 4 °C. Integrity of single cell suspensions was confirmed by an FSC/SSC analysis performed by flow cytometry (not shown).

Cell Migration Assay Cell migration assay experiments were carried out using the Real-Time Cell Analyzer (RTCA) Dual-Plate (DP) Instrument of the xCELLigence system (Roche Diagnostics). Cells were trypsinized and 20,000 cells/well were seeded onto CIM-Plates 16 (Roche Diagnostics). Isolated mouse EC was seeded in the wells immediately after their extraction. These migration plates are similar to conventional Transwells (8 μm pore size) but with gold electrode arrays on the bottom side of the membrane to provide a real-time measurement of cell migration. Prior to cell seeding, the underside of the wells from the upper chamber was coated with 25 μL of 0.15% gelatin in PBS and incubated for 1 h at 37 °C. S1P or LPA were dissolved in serum-free culture medium and added to the lower chamber. Cell migration was monitored for 6 h. The impedance values were measured by the RTCA DP Instrument software and were expressed in arbitrary units as Normalized Cell Migration Index. Each experiment was performed three times in triplicate.

Immunoblotting Procedures Cells were lysed in a buffer containing 1 mM each of NaF and Na_3VO_4, and proteins from control and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.1% Tween-20 (TBST). Membranes were further washed in TBST and incubated 1 h with primary antibodies (1/1000 dilution) in TBST containing 3% BSA and 0.05% NaN_3. The primary antibody was removed by washing with TBST, followed by a 1 h incubation with horseradish peroxidase-conjugated anti-mouse IgG (1/5000 dilution) in TBST containing 5% non-fat dry milk. Immunoreactive material was visualized by enhanced chemiluminescence (Amersham Biosciences, Baie d'Urfé, QC).

Transfection Method and RNA Interference HBMEC were transiently transfected with an equimolar (20 nM) mixture of three different human siRNA (Qiagen) against C/EBPβ (Hs_CEBPB_4 FlexiTube siRNA: SI00073640; Hs_CEBPB_5 FlexiTube siRNA: SI02777292; Hs_CEBPB_7 FlexiTube siRNA: SI03058062) in order to achieve maximal gene repression. Single siRNA sequences were used for LRP1 (Hs_LRP1_9 FlexiTube siRNA: SI05113192), S1P1 (Hs_EDG1_1 FlexiTube siRNA: SI00376201), S1P3 (Hs_EDG3_5 FlexiTube siRNA: SI02757391) gene silencing or scrambled sequences (AllStar Negative Control siRNA, 1027281) using Lipofectamine 2000 (ThermoFisher Scientific, Burlington, ON). Specific gene knockdown was evaluated by qRT-PCR as described below. The m4LRP1 cDNA plasmid was kindly provided by Dr. Guojun Bu (Washington University School of Medicine, St Louis, MO).

Total RNA Isolation, cDNA Synthesis, and Real-Time Quantitative RT-PCR Total RNA was extracted from HBMEC monolayers or from isolated mouse EC using TriZol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 1 μg of total RNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Gene expression was quantified by real-time quantitative PCR using SsoFastTM EvaGreen® Supermix (Bio-Rad, Hercules, CA). DNA amplification was carried out using a CFX Connect Real-Time System (Bio-Rad, Hercules, CA) and product detection was performed by measuring binding of the fluorescent dye SsoFastTM EvaGreen® to double-stranded DNA. The following QuantiTect human and mouse primers were obtained from Qiagen (Valencia, CA): LRP1 (QT00025536), S1P1 (QT00208733), S1P2 (QT00230846), S1P3 (QT00244251), S1P4 (QT01192744), S1P5 (QT00234178), CEBPB (QT00237580), LRP1 (QT00155981), S1P1 (QT00243628), S1P2 (QT00262773), S1P3 (QT00132160), S1P4 (QT00260141), S1P5 (QT00282744), CEBPB (QT00320313). The relative quantities of target gene mRNA were compared against human GAPDH (QT00079247) and PPIA (QT01866137) or mouse GAPDH (QT01658692) and PPIA (QT00247709) internal mRNA controls, and were measured by following a ΔCt method employing an amplification plot (fluorescence signal vs. cycle number) and obtaining a cycle threshold. The difference (ΔCt) between the mean values in the triplicate samples of the target gene and those of GAPDH and PPIA mRNAs were calculated by CFX ManagerTM Software version 3.1 (Bio-Rad, Hercules, CA) and the normalized expression (ΔΔCt) was quantified.

Statistical Data Analysis Data are representative of three or more independent experiments. Statistical significance was assessed using GraphPad Prism 5 software with Student's paired *t* test. $*P < 0.05$ was considered significant.

Results

C/EBPβ Contributes to S1P Receptor Transcriptional Control in Human Brain Microvascular Endothelial Cells The interconvertible bioactive sphingolipids sphingosine-1-phosphate (S1P) and ceramide have profound effects on GBM cells, with S1P regulating cell survival, proliferation and invasion [6]. Although current evidence supports

A

Transcription factors	S1P receptor promoter sequences				
	S1P1	S1P2	S1P3	S1P4	S1P5
AR	1				
PRA	1				
PRB	1				
c-Ets-1	1				1
GATA-1	1				3
STAT4	1	2			
FOXP3	1	2	2		
RXRα	2			2	1
ATF-2		1			
c-Jun		1		1	
GCF		1	1		
EIK-1			1		
C/EBPα			1		
NF1			2		
AP-2αA	1	3	1	2	2
ER-α	1	3	1	6	3
Pax-5	1	4	3	11	10
YY1	2	4	4	5	9
GRβ	2	6	2	3	2
GRα	2	9	5	5	4
C/EBPβ	13	18	15	13	14
GR	1	4	1		1
TFII-1	3	8	1		2
TFIID	3	1	1		1
p53				3	3
IRF-2				1	1
XBP-1				1	
HNF3α				1	
E2F-1					1

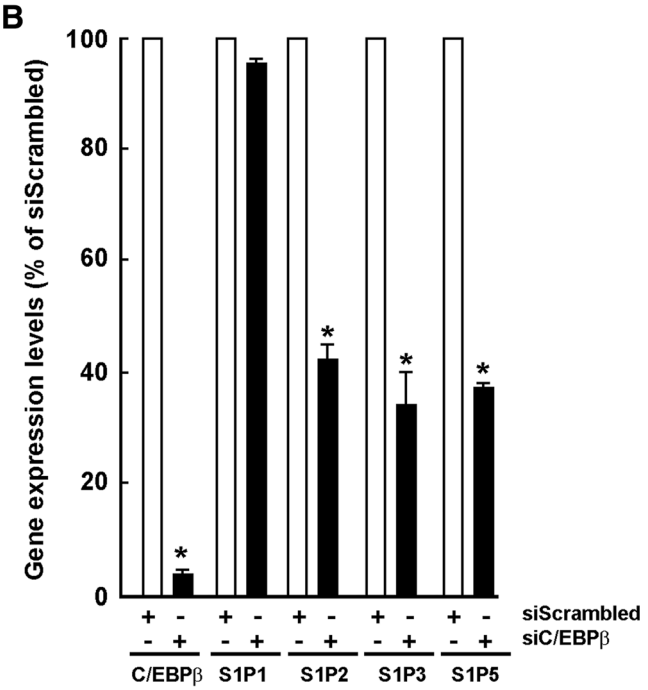

Fig. 1 C/EBPβ contributes to S1P receptors transcriptional control in human brain microvascular endothelial cells. **a** The presence and frequency of various transcription factor binding sites within promotor sequences of all five S1P receptors was obtained as described in the Methods section. Each transcription factor regulatory sequence with a dissimilarity rate of 0% was assessed. C/EBPβ potential binding sites (highlighted in gray) appeared with the highest frequency in all five S1P receptor promoters. **b** Transient gene silencing of *C/EBPβ* (siC/EBPβ, black bars) was performed in transfected HBMEC using specific siRNAs as described in the "Experimental Procedures" section, and gene expression of *C/EBPβ*, *S1P1*, *S1P2*, *S1P3*, and *S1P5* were assessed by qRT-PCR (*S1P4* is not expressed in HBMEC, not shown). Normalized gene expression with respect to *GAPDH* and *PPIA* is shown with the means ± SEM of three independent experiments. $^*P < 0.05$

a role for S1P signaling at the BBB [22], little is known about the vascular plasticity and transcriptional adaptive mechanisms involved in oncogenic transformation [23]. A better understanding of the regulation of cerebrovascular S1P receptor

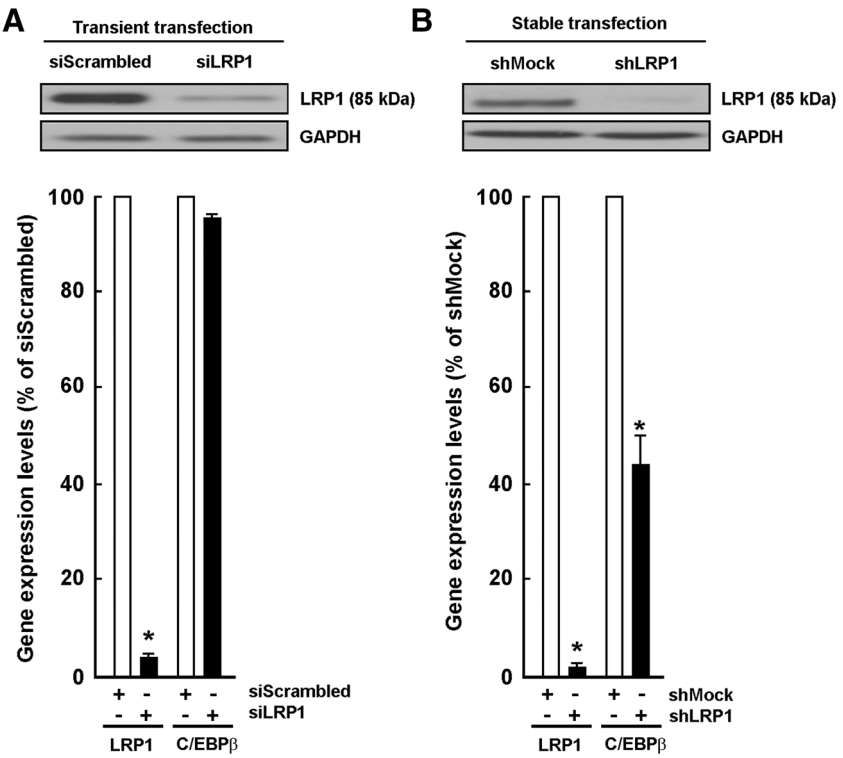

Fig. 2 Adaptive decrease in *C/EBPβ* gene expression is consequent to stable, but not transient, LRP1 repression in human brain microvascular endothelial cells. LRP1 silencing was performed in HBMEC either **a** transiently (siLRP1) or **b** stably (shLRP1) as described in the Experimental Procedures section. *LRP1* and *C/EBPβ* gene expression levels were assessed by qRT-PCR, whereas LRP1 protein expression was assessed by Western Blot as described in the Methods section and compared to their siScrambled or shMock controls. Normalized *LRP1* and *C/EBPβ* gene expression with respect to *GAPDH* and *PPIA* is shown with the means ± SEM of three independent experiments. $^*P < 0.05$

expression and of S1P receptor-mediated signaling pathways may therefore offer potential anti-angiogenic therapeutic targeting strategies for GBM. In order to predict the transcription factor binding sites present within the promoter sequences of the five human S1P receptors known, we used the Ensembl Genome Browser tool coupled to the PROMO algorithm [24–26]. Our in silico screen of the human genetic sequences for these five genes enabled us to identify putative transcription factor binding sites with a dissimilarity rate of less than 15% [27]. Based on these findings, we extracted each promoter sequence by annotating all transcription factor sequences with a 0% dissimilarity rate. We noticed a high frequency of CCAAT/Enhancer binding protein β (C/EBPβ) regulatory sequences within all five receptors of S1P (Fig. 1a), suggesting a crucial role may be played by this transcription factor in regulating S1P receptors transcription. Transient gene silencing of C/EBPβ was performed and it significantly decreased *S1P2*, *S1P3*, and *S1P5* gene expression, whereas *S1P1* expression remained unaffected (Fig. 1b). *S1P4* basal gene expression was undetectable in HBMEC (data not shown).

Adaptive Decrease in C/EBPβ Gene Expression Is Consequent to Stable, But Not Transient, LRP1 Repression in Human Brain Microvascular Endothelial Cells LRP1 was recently ascribed a role in angiogenesis, and represents an unexpected link between lipoprotein receptors and sphingolipid signaling with potential implications in tumor angiogenesis and inflammatory processes [17]. Since LRP1 is highly expressed in human brain microvessels [28], we sought to address whether any signaling crosstalk linked LRP1 to cerebrovascular S1P receptor transcriptional control involving C/EBPβ. For this purpose, LRP1 gene and protein expressions were repressed either transiently (siLRP1) using siRNA approaches (Fig. 2a), or stably via the generation and

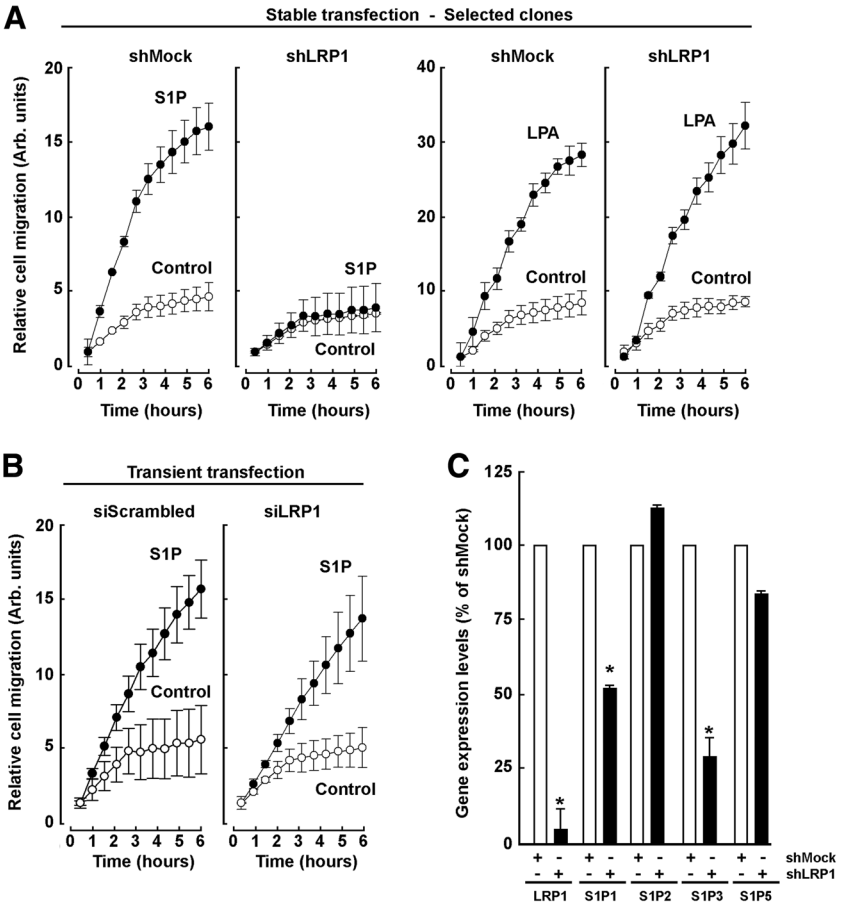

Fig. 3 Stable, but not transient, LRP1 repression prevents S1P-induced HBMEC migration. Cells were seeded into the chambers of an xCELLigence system as described in the Methods section. Cells were then primed with either vehicle (0.1% MeOH for the S1P stimulations, or 0.1% PBS-BSA for LPA stimulations), 1 μM S1P or 2 μM LPA. Migration was measured in real-time for 6 h. **a** Migration of control HBMEC (shMock) and HBMEC where LRP1 was stably repressed (shLRP1) was performed in the absence (open circles) or presence of S1P/LPA (closed circles). **b** Transient control (siScrambled) or LRP1 gene repression (siLRP1) was performed in HBMEC as described in the "Experimental Procedures" section. Migration was assessed in response to vehicle (Control, *open circles*) or 1 μM S1P (*closed circles*). **c** Gene expression levels of *LRP1*, *S1P1*, *S1P2*, *S1P3* and *S1P5* were assessed by qRT-PCR in shMock (*white bars*) and in shLRP1 (*black bars*) HBMEC. Normalized gene expression over GAPDH and PPIA is shown with the means ± SEM of three independent experiments. *$P < 0,05$

selection of an HBMEC clone in which *LRP1* knockdown was generated through shRNA strategies (shLRP1, Fig. 2b). Both led to very significant decreases in LRP1 gene and protein expression as demonstrated through qRT-PCR (Fig. 2, lower panels) and immunoblotting (Fig. 2, upper panels). While C/EBPβ expression was unaltered upon transient siLRP1 repression (Fig. 2a), significant downregulation of *C/EBPβ* was observed in shLRP1 (Fig. 2b). This suggests that long-term reprogramming mechanisms are involved in *C/EBPβ* transcriptional regulation in HBMEC and consequent to constitutive shLRP1-mediated lack of function. We next addressed whether long-term LRP1 repression further affected functional S1P receptor-mediated response.

Stable, But Not Transient, LRP1 Repression Prevents S1P-Induced HBMEC Migration Both LRP1 and S1P receptor-mediated molecular signaling are known to be involved in invasion and angiogenesis processes [29–31]. In order to investigate the molecular link between LRP1 expression and the S1P receptor-mediated response, we assessed HBMEC migration in real-time using the xCELLigence system as described in the "Experimental Procedures" section. We found that shLRP1-transfected HBMEC became unresponsive to S1P, as compared to control HBMEC (shMock, Fig. 3a), whereas transient siLRP1 gene silencing did not affect HBMEC responsiveness to S1P (Fig. 3b). Chemotactic response to lysophosphatidic acid (LPA), another natural bioactive lysophospholipid which signals through defined GPCRs [32], was found unaffected (Fig. 3a). Since stable repression of LRP1 altered C/EBPβ, a transcription factor that regulates S1P receptor transcription (Fig. 1a), we next assessed the gene expression levels of the S1P receptors *S1P1*, *S1P2*, *S1P3*, and *S1P5* in shLRP1-transfected cells where LRP1 was stably repressed. We found that *S1P1* and *S1P3* expression was downregulated, while *S1P5* was unchanged and *S1P2* tended to increase (Fig. 3c). The downregulated *S1P1* and *S1P3* receptors are therefore candidates explaining, in part, why S1P chemotactic response is altered in cells upon constitutive repression of LRP1.

Lack of Response to S1P in Endothelial Cells Isolated from Brains of Lrp1(EC)$^{-/-}$ Mice We next examined the effect of LRP1 repression on S1P-mediated chemotactism using freshly isolated EC from brains of *Lrp1*(EC)$^{-/-}$ mice

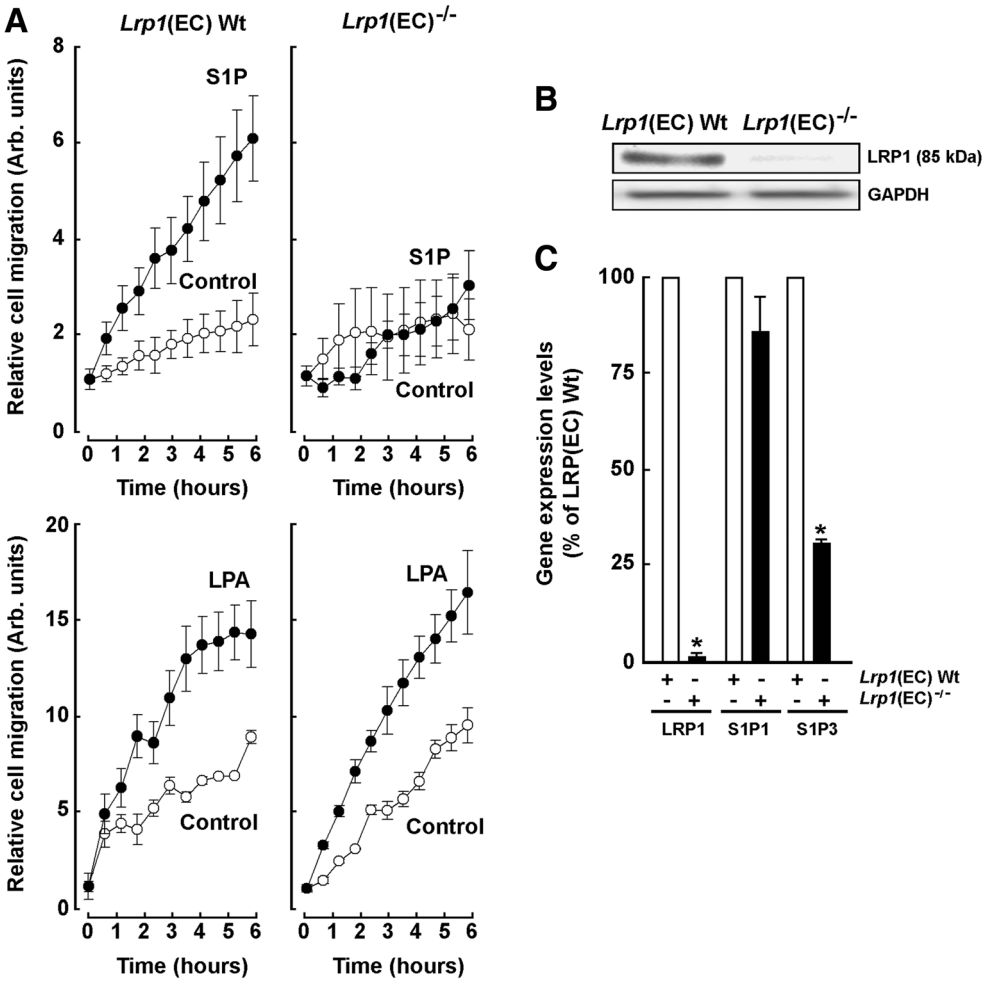

Fig. 4 Lack of response to S1P in brain endothelial cells isolated from *Lrp1*(EC)$^{-/-}$ mouse. **a** Endothelial cells were isolated from *Lrp1*(EC) Wt or LRP1-deficient (*Lrp1*(EC)$^{-/-}$) mouse brains as described in the "Experimental Procedures" section, then seeded into the chambers of the xCELLigence system. Cell migration was then monitored in response to vehicle (0.1% MeOH for the S1P stimulations, or 0.1% PBS-BSA for LPA stimulations), 1 μM S1P or 2 μM LPA. **b** Immunoblotting of cell lysates was performed to detect LRP1 expression in the endothelial cells from both animal models. Coomassie blue staining was performed to show equal loading of proteins. **c** Gene expression levels of *LRP1*, *S1P1*, and *S1P3* were assessed by qRT-PCR in *Lrp1*(EC) Wt (*white bars*) and *Lrp1*(EC)$^{-/-}$ (*black bars*) murine brain endothelial cells. Normalized gene expression with respect to *GAPDH* and *PPIA* is shown with the means ± SEM of three independent experiments. *$P < 0.05$

and compared it to EC isolated from Wt-*Lrp1*(EC) mice brains. In vitro cell migration analysis was performed to compare LRP1 repression effects between freshly isolated cells from brain tissues and engineered stable transfectant cells. We observed closely matched in vitro effects between our stable shLRP1-transfected cell model, with regards to S1P stimulation, and brain EC isolated from *Lrp1*(EC)$^{-/-}$ mice lacked LRP1 (Fig. 4b) which were also less responsive to S1P than were Wt cells (Fig. 4a, upper panels). The response to LPA remained identical between the Wt- and *Lrp1*(EC)$^{-/-}$ migration (Fig. 4a, lower panels). While the *S1P1* and *S1P3* receptors were downregulated in vitro upon shLRP1 stable repression, only *S1P3* expression was significantly decreased in EC isolated from *Lrp1*(EC)$^{-/-}$ mice (Fig. 4c). Altogether, these in vivo results strengthen the crosstalk concept between LRP1 signal transducing functions and S1P receptor-mediated signaling within an adaptive response to constitutive shRNA-mediated repression of LRP1. The identity of the specific signaling pathway involved was next investigated.

LRP1 Regulates S1P Receptor-Mediated Signaling and Requires Activation of p38 MAPK and ERK Downstream Signaling Pathways Mitogen-activated protein kinases (MAPK) represent good candidates for the downstream signaling of LRP1 and S1P because they both regulate multiple cellular processes such as gene expression, proliferation, invasion and migration, metabolism and apoptosis [33]. Western blot analysis of the phosphorylation status of three major MAPK downstream signals was performed and confirmed the involvement of p38 MAPK, ERK1/2, and JNK in response to S1P-mediated signaling as all became maximally phosphorylated after ~10–20 min (Fig. 5a). When stable shLRP1-transfected HBMEC were stimulated with S1P, only JNK remained phosphorylated while p38 MAPK and ERK1/2's phosphorylation status significantly decreased (Fig. 5b). Our data confirm that adaptive mechanisms take place in response to constitutively repressed LRP1 expression, which prevent adequate MAPK pathway activation and which lead to HBMEC inability to migrate under S1P stimulation.

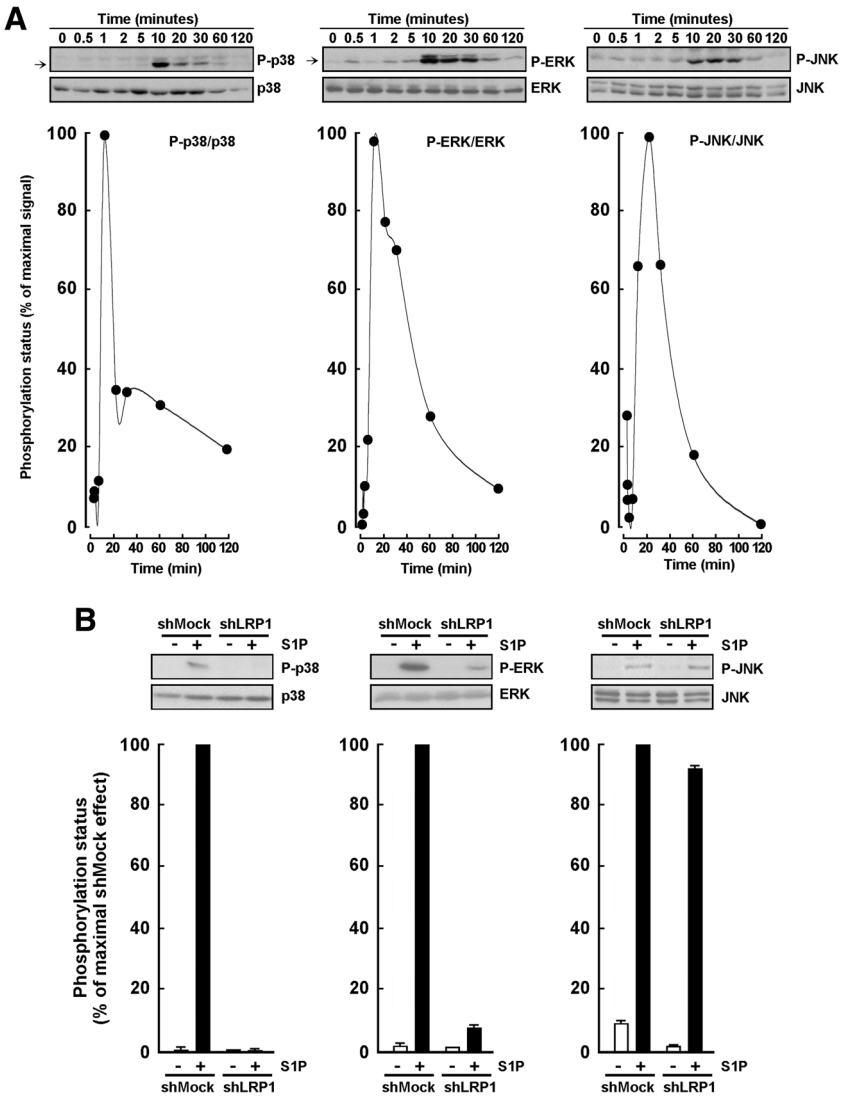

Fig. 5 LRP1 modulation of S1P receptor-mediated signaling requires activation of p38 MAPK and ERK downstream signaling pathways. **a** Phosphorylation status of p38, ERK1/2, and JNK proteins were assessed in 20 μg lysates extracted from HBMEC submitted to 1 μM S1P stimulation for up to 120 min (*upper panels*, an *arrow* indicates which immunoreactive band to analyze). A representative scanning densitometry quantification is shown for each phosphorylated intermediate and expressed as the ratio of phosphorylated/total protein signal. **b** Phosphorylation status of p38, ERK1/2, and JNK in response to S1P was compared between control HBMEC (shMock) or in which LRP1 expression was stably repressed (shLRP1). A representative scanning densitometry quantification is shown for each phosphorylated intermediate and expressed as the % of maximal phosphorylated/total protein signal from shMock HBMEC

S1P3 Is Required for Functional S1P-Mediated Migration of HBMEC S1P signaling is known to activate different pathways through its five receptors leading to migration and angiogenesis [31]. Given that *S1P1* and *S1P3* expressions were initially found to be decreased upon the absence of LRP1, and that stable shLRP1mediated repression resulted in HBMEC which lacked S1P chemotactic cell response, we next investigated which of the S1P1 or S1P3 receptors is involved in the repressed S1P-mediated cell migration. Using transient siRNA gene silencing of *S1P1* and *S1P3* (Fig. 6b), we found that only S1P3 was required in order for HBMEC to migrate efficiently (Fig. 6a). Furthermore, we also observed that the p38 MAPK and ERK1/2 signaling pathways were completely repressed when *S1P3* was transiently silenced (Fig. 6c) demonstrating its importance for the angiogenic response of HBMEC.

Overexpression of LRP1 Cluster IV Upregulates S1P3 Transcription and Restores S1P-Mediated Cell Migration in shLRP1 HBMEC In order to delineate the minimal LRP1 structural requirement necessary to ensure an adequate S1P-mediated response, we transfected cells and transiently overexpressed recombinant LRP1 cluster IV (m4LRP1) in shLRP1 HBMEC. Recombinant m4LRP1 anchors to the plasma membrane and provides access to a short intracellular cytoplasmic domain [34]. Transfection efficacy of the m4LRP1 cDNA plasmid was confirmed both at the protein (Fig. 7a, upper panels) and gene (Fig. 7a, histogram gray bar) expression levels, and found to rescue S1P-mediated cell migration in shLRP1 HBMEC (Fig. 7b) to levels comparable to those of control (shMock) HBMEC. Since constitutive repression of LRP1 leads to diminished S1P1 and S1P3 transcription, we next determined whether m4LRP1 overexpression enabled any transcriptional control of these two genes. We found that, while *S1P1* transcript levels remained low, those of *S1P3* were significantly rescued in m4LRP1-transfected shLRP1 HBMEC (Fig. 7c). This observation suggests that *S1P3* is under the direct transcriptional control of LRP1, whereas *S1P1* possibly required an additional level of control in addition to LRP1 itself.

Discussion

In this study, we highlight an original signaling axis linking the LRP1 intracellular domain's capacity to regulate gene

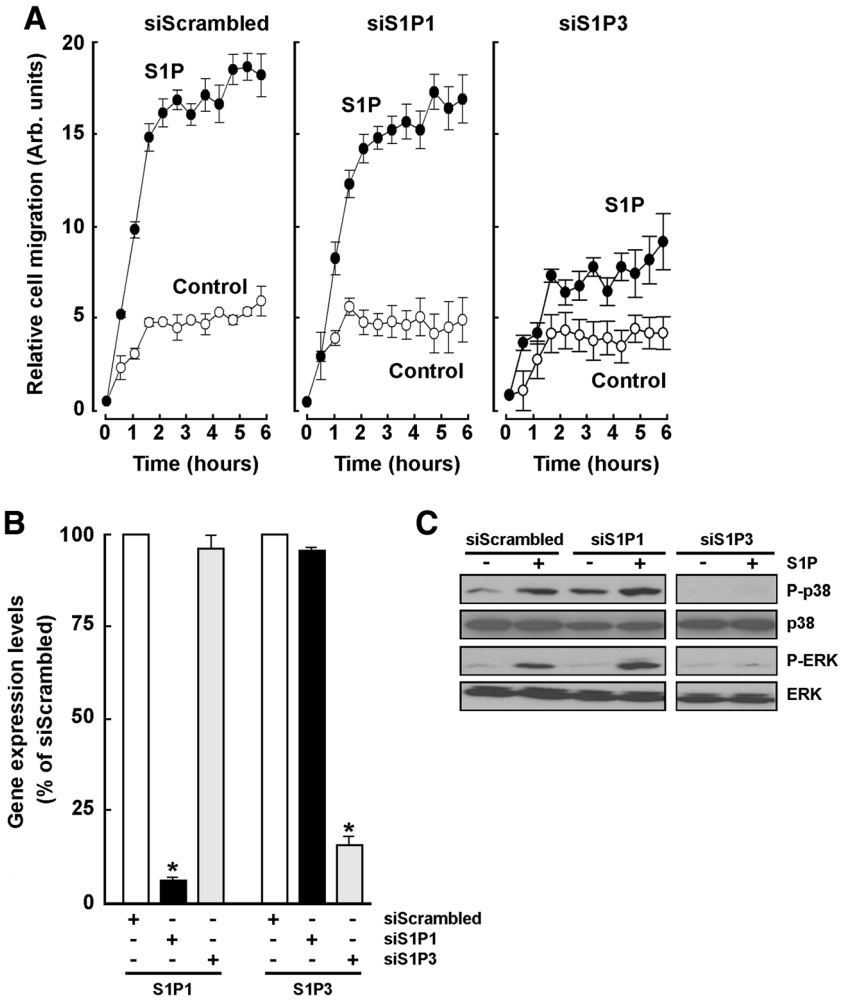

Fig. 6 S1P3 is required for proper S1P-mediated signaling and migration of HBMEC. **a** Transient control (siScrambled), S1P1 (siS1P1), or S1P3 (siS1P3) gene silencing were performed in HBMEC as described in the Methods section. Cells were left to recuperate for 24 h, seeded into the chambers of the xCELLigence system, and primed with either vehicle (0.1% MeOH, control *open circles*) or 1 μM S1P (*closed circles*). Migration was measured in real-time for 6 h. **b** Validation and efficacy of S1P1 (*black bars*) and S1P3 (*gray bars*) gene silencing was performed by qRT-PCR. Normalized gene expression over GAPDH and PPIA is shown with the means ± SEM of three independent experiments. $^*P < 0.05$. **c** Phosphorylation status of p38 and of ERK1/2 was performed by immunoblotting lysates following 10 min S1P treatment. A representative Western blot is shown

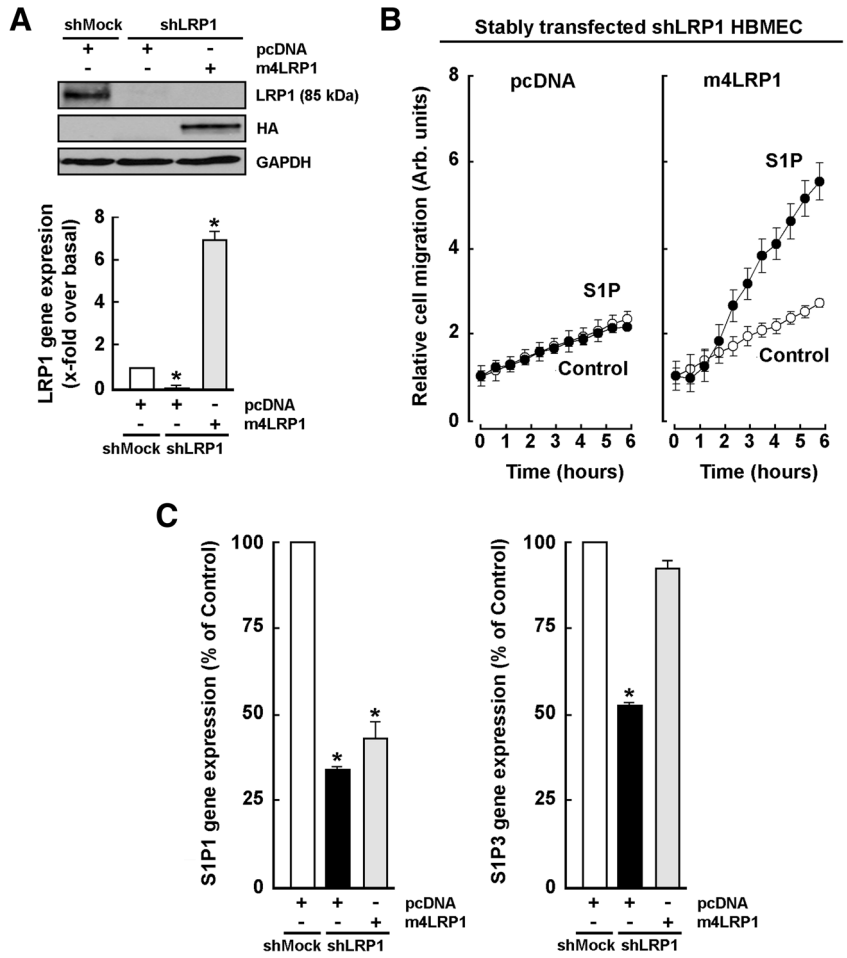

Fig. 7 Overexpression of m4LRP1 (cluster 4) upregulates S1P3 transcription and restores S1P-mediated cell migration in shLRP1-transfected HBMEC. **a** Stable LRP1-repressed HBMEC (shLRP1) were transiently transfected with pcDNA or with a cDNA plasmid encoding HA-tagged LRP1 cluster IV (m4LRP1) as described in the "Experimental Procedures" section. Cell lysates were used for Western blot immunodetection of GAPDH, endogenous 85 kDa LRP1, and HA-tagged m4LRP (*upper panels*). *LRP1* gene expression was performed by qRT-PCR. **b** Real-time cell migration was assessed for shLRP1 HBMEC transfected with pcDNA or with m4LRP1 in response to vehicle (control, *open circles*) or S1P (*closed circles*) using an xCELLigence system. **c** *S1P1* and *S1P3* gene expression were assessed by qRT-PCR

transcription to sphingolipid signaling within the cerebrovascular compartment. More specifically, we identified the S1P receptor S1P3 as an important downstream actor in the adaptive mechanisms consequent to a constitutive lowering of LRP1 expression. Whether such metabolic reprogramming involves LRP1 activities, separate from ligand transport or intracellular trafficking/endocytosis, remains to be confirmed. LRP1 is already known to regulate signal transduction and to interact with other cell surface membrane receptors such as PDGFRβ and β2-integrins [35, 36]. While cell surface LRP1 regulation of specific S1P receptors can be envisioned, it can effectively be ruled out since transient LRP1 silencing did not alter the brain EC response to S1P. Moreover, overexpression of a partial recombinant LRP1 encompassing its transmembrane and intracellular domains was found sufficient to reverse the lack of response to S1P and to rescue *S1P3* transcription. Thorough in silico analysis of S1P receptor promoter sequences revealed several potential transcription factor binding sites possibly involved in the regulation of S1P receptors gene expression. Among these, a C/EBPβ regulatory sequence was repeatedly found within all five S1P receptors promoter. Transient silencing of *C/EBPβ* confirmed that its gene product was involved and specifically regulated *S1P2*, *S1P3*, and *S1P5* transcription, and its expression was also significantly diminished in HBMEC where LRP1 was stably repressed. Collectively, our in vitro and in vivo evidence clearly confirms that adaptive mechanisms, consequent to LRP1-mediated metabolic reprogramming, alter the brain EC angiogenic response (See summarizing scheme in Fig. 8).

Physiological cellular responses to S1P are associated with angiogenic and pro-inflammatory processes. In fact, S1P is highly synthesized and secreted following increased SphK activity, as observed within several solid tumor cancers, and able to trigger angiogenesis [37, 38]. Given that S1P can also be released from platelets and immune cells such as monocyte-derived macrophages and neutrophils [39], pro-inflammatory paracrine signaling may also be triggered by S1P in order to promote cell death/survival signaling or cell migration and proliferation [40]. Incidentally, all these events can take place within the hypoxic tumor microenvironment. As such, low oxygen tension was found to augment the outgrowth of EC sprouting and directed migration in response to S1P [41]. Recently, the SphK/S1P signaling pathway was found to elicit various cellular processes including cell proliferation, cell survival or angiogenesis [42]. Hypoxia also

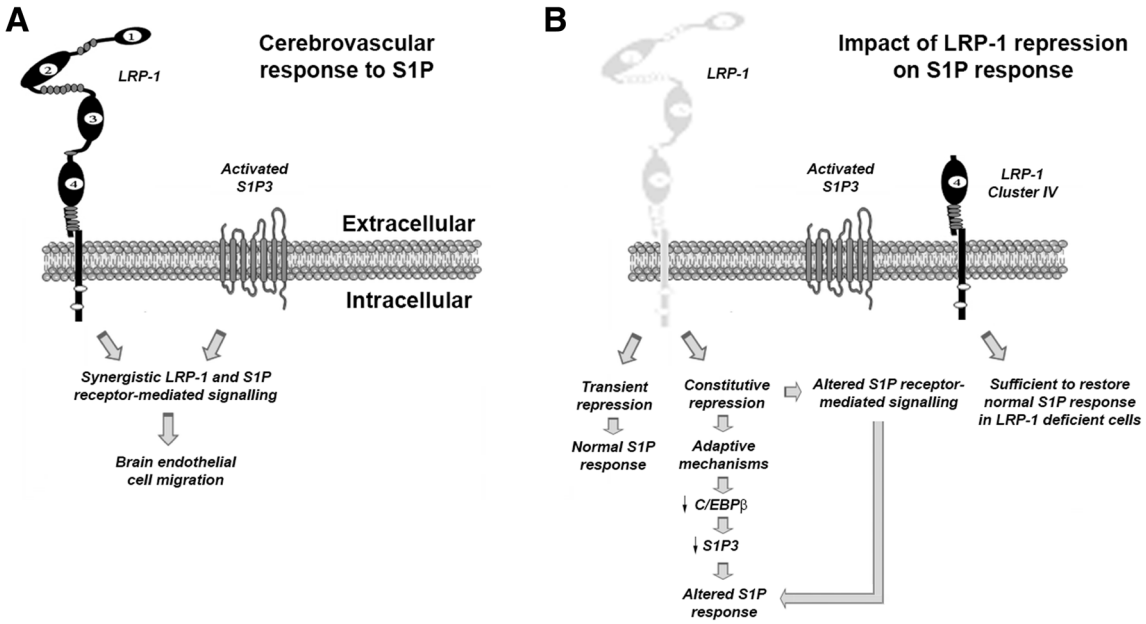

Fig. 8 Summarizing scheme of the synergistic LRP-1 and S1P receptor-mediated signaling on cerebrovascular response. **a** LRP-1 is depicted to synergize with S1P-activated S1P3 receptor for proper intracellular signaling, which leads to increased brain endothelial cell migration. Physiological sources of S1P can originate from platelets, whereas pathophysiological sources of S1P may originate from glioblastoma cells which possess high levels of sphingosine kinase activity. **b** Transient LRP-1 repression does not appear to alter brain endothelial cells response to S1P, whereas constitutive LRP-1 repression triggers adaptive transcriptional mechanisms involving C/EBPβ which ultimately leads to altered response to S1P. Overexpression of the membrane-anchored cluster IV domain of LRP-1 is sufficient to restore normal S1P response and suggests a crucial cell signaling role for the intracellular domain of LRP-1

upregulated LRP1 expression in human vascular smooth muscle cells through HIF-1α induction [43]. Combined targeting of SphK/S1P signaling and LRP1 functions may thus represent an attractive strategy for therapeutic intervention in cancer.

Metabolic reprogramming is also associated with crucial S1P signaling which can dictate stem cell differentiation status. In fact, S1P was found to reprogram mesenchymal stem cells (MSC) towards osteogenic differentiation and to inhibit adipogenic differentiation [44]. Interestingly, a Gi-dependent S1P signaling was found to suppress C/EBPβ expression essential for adipogenic differentiation [44]. As we have shown that transcription of all five S1P receptors is potentially regulated by C/EBPβ, a transcription factor of the basic-leucine zipper (bZIP) class having a role in the CNS, one may consider a possible retroactive mechanism involved in general physiological processes in EC such as proliferation [45], cell death and survival, energy metabolism and inflammation [46]. Given that stable repression of LRP1 leads to diminished C/EBPβ expression and to altered S1P-mediated HBMEC migration, our data suggest that the in vivo and in vitro long-term expression status of lipoprotein receptors such as LRP1 can lead an adaptive transcriptional machinery to affect the growth factor- and cytokine-mediated signaling that controls EC migration and angiogenesis.

Pro-angiogenic intracellular signal transducing pathways can regulate cell migration by both paracrine and autocrine means [47]. The S1P pathway has been shown to require Rho small GTPases and the PI3K pathways and to modify cytoskeletal activity while the paxillin/focal adhesion kinase (Pax/FAK) signaling module triggers adhesion [48]. S1P can also activate GPCRs through Ras and Raf, subsequently activating MAPK pathways to potentially regulate migration [49]. Hence, we focused on MAPK pathways since they are directly associated with the migration process. We found that LRP1 was required for S1P-mediated ERK1/2 and p38 MAPK activation, but not for JNK activation. This evidence supports the existence of a signaling crosstalk involving LRP1 and S1P in the migratory process which could represent a promising anti-angiogenic target for preventing brain tumor neovascularization. Moreover, it has been reported that MAPK pathways can modulate and phosphorylate C/EBPβ for further gene transcription [50], confirming its role as a candidate feedback transcription factor for differentially expressed S1P receptors in stable shLRP1-transfected HBMEC.

HBMEC were found to express only four of the five S1P receptor transcripts. When differential gene expression profiling was performed between transient and stable LRP1 knockdowns, in vitro and in vivo LRP1 silencing strategies both showed that S1P3 was the primary S1P receptor involved in the altered adaptive response to S1P upon LRP1 repression. Some of the strongest evidence regarding LRP1's role in regulating *S1P3* transcription was provided

upon overexpression of LRP1 cluster IV (m4LRP1). Constitutive cell surface anchoring of these transmembrane and cytoplasmic domains of LRP1 was found to significantly rescue *S1P3* transcript levels. As previous observations have indicated roles for S1P1 and S1P3 in the chemotactic vascular and glial migration towards S1P [51, 52], this suggests that migration can mostly be activated through Gi and possibly $G_{12/13}$ signaling, consistent with a decrease in ERK and p38 phosphorylation.

Regulation of LRP1 expression and function during oncogenic transformation may also be performed through alternate mechanisms. For instance, the membrane type-1 matrix metalloproteinase MT1-MMP, highly expressed in several cancers [53], has been shown to cleave LRP1 at the cell surface, which is believed to result in an increased invasive phenotype through increases in global ECM hydrolytic activity [54]. In fact, high expression of MT1-MMP has been correlated with decreased expression of LRP1 in advanced stages of Wilms tumors [55]. Whereas shedding of the extracellular domain of LRP1 has been reported to impact on its extracellular domain and to functionally decrease the recycling of LRP1 ligands [56], the functions of the remaining LRP1 transmembrane and cytoplasmic domains may still be unaltered and sufficient for transducing S1P-mediated signaling. Interestingly, MT1-MMP/S1P signaling crosstalk has also been recently documented in bone marrow stromal cells [57, 58], glioblastoma cells [59], and CD133+ glioblastoma stem cells [60]. Whether LRP1 modulates any of this signaling remains to be confirmed. Pharmacological inhibition of S1P signaling by EGCG was also found to inhibit differentiation of promyelomonocytic leukemia cells by PMA [61]. EGCG is an anti-angiogenic and anti-inflammatory green tea-derived catechin which has been repeatedly documented as inhibiting MT1-MMP catalytic and signaling functions [62, 63].

In conclusion, our study provides evidence for significant angiogenic reprogramming consequent to long-term adaptive mechanisms taking place within cerebrovascular EC upon LRP1 repression. Targeting of the LRP1/S1P signaling axis at the BBB should be considered for future antiangiogenic therapeutic strategies. S1P receptors expression could also hold promise as new cerebrovascular biomarkers in the design and development of targeted new drugs.

BBB, blood-brain barrier; C/EBPβ, CCAAT/enhancer binding protein β; EC, endothelial cells; GBM, glioblastoma; HBMEC, human brain microvascular endothelial cells; LPA, lysophosphatidic acid; LRP1, low-density lipoprotein receptor-related protein 1; MAPK, mitogen-activated protein kinases; MSC, mesenchymal stem cells; MT1-MMP, membrane type-1 matrix metalloproteinase; S1P, sphingosine-1-phosphate; SphK, sphingosine kinase.

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Authors' Contributions AV, BA conceived and coordinated the study and wrote the paper. AV performed and analyzed all the experiments. CC generated the stable shLRP1 knockdown HBMEC. AZ technical assistance for small animal work and contributed to the preparation of the figures. All authors reviewed the results and approved the final version of the manuscript.

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