Biochemical and Biophysical Research Communications 497 (2018) 32–38

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

GPR182 is a novel marker for sinusoidal endothelial differentiation with distinct GPCR signaling activity in vitro

Christian David Schmid [a, b], Kai Schledzewski [a, b], Carolin Mogler [c, d], Nina Waldburger [d, e], Viktoria Kalna [f], Alexander Marx [b, g], Anna Maria Randi [f], Cyrill Géraud [a, b], Sergij Goerdt [a, b], Philipp-Sebastian Koch [a, b, *]

[a] Department of Dermatology, Venereology and Allergology, University Medical Center and Medical Faculty Mannheim, University of Heidelberg, Center of Excellence in Dermatology, Mannheim, Germany
[b] European Center for Angioscience, Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany
[c] Institute of Pathology, Technical University of Munich, Munich, Germany
[d] Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany
[e] Liver Cancer Center Heidelberg, University Hospital Heidelberg, Heidelberg, Germany
[f] Vascular Sciences, Imperial Centre for Translational and Experimental Medicine, National Heart and Lung Institute, Imperial College London, London, United Kingdom
[g] Institute of Pathology, University Medical Center and Medical Faculty Mannheim, University of Heidelberg, Mannheim, Germany

ARTICLE INFO

Article history:
Received 25 January 2018
Accepted 30 January 2018
Available online 1 February 2018

Keywords:
Liver sinusoidal endothelial cells (LSEC)
GPCR
Endothelial heterogeneity
Hepatocellular carcinoma (HCC)
Liver fibrosis

ABSTRACT

Endothelial cells (EC) along the vascular tree exhibit organ-specific angiodiversity. Compared to most other ECs, liver sinusoidal endothelial cells (LSEC) that constitute the organ-specific microvasculature of the liver are morphologically and functionally unique. Previously, we showed that transcription factor Gata4 acts as a master regulator controlling LSEC differentiation. Upon analysis of the molecular signature of LSEC, we identified GPR182 as a potential LSEC-specific orphan G-protein coupled receptor (GPCR). Here, we demonstrate that GPR182 is expressed by LSEC and by EC with sinusoidal differentiation in spleen, lymph node and bone marrow in healthy human tissues. In a tissue microarray analysis of human hepatocellular carcinoma (HCC) samples, endothelial GPR182 expression was significantly reduced in tumor samples compared to peritumoral liver tissue samples ($p = 0.0105$). Loss of endothelial GPR182 expression was also seen in fibrotic and cirrhotic liver tissues. In vitro, GPR182 differentially regulated canonical GPCR signaling pathways as shown using reporter luciferase assays in HEK293T cells. Whereas ERK and RhoA signaling were inhibited, CREB and Calcium signaling were activated by ectopic GPR182 overexpression. Our data demonstrate that GPR182 is an endothelial subtype-specific marker for human sinusoidal EC of the liver, spleen, lymph node and bone marrow. In addition, we provide evidence for GPR182-dependent downstream signaling via ERK and SRF pathways that may be involved in regulating endothelial subtype-specific sinusoidal differentiation and sinusoidal functions such as permeability.

1. Introduction

Endothelial cells (EC) along the vascular tree exhibit organ-specific angiodiversity which has led to the classification of continuous, fenestrated and sinusoidal endothelium. Sinusoidal endothelial cells represent a highly specific type of microvascular endothelium with a unique morphology such as fenestrations without diaphragms and lack of a basement membrane and they are found in liver, spleen, bone marrow, lymphoid tissue and endocrine organs [1]. Liver sinusoidal endothelial cells (LSEC) that constitute the microvasculature of the liver exert important regulatory functions on local and systemic levels such as scavenger functions [2], the regulation of the portal blood pressure and the

Abbreviations: EC, endothelial cells; LSEC, liver sinusoidal endothelial cells; GPCR, G protein coupled receptor; HCC, hepatocellular carcinoma; LMEC, lung microvascular endothelial cells; ISC, intestinal stem cells; TMA, tissue microarray; RFP, red fluorescent protein.

* Corresponding author. Klinik für Dermatologie, Venerologie und Allergologie, Universitätsmedizin Mannheim, D-68135 Mannheim, Germany.
E-mail address: philipp.koch@umm.de (P.-S. Koch).

https://doi.org/10.1016/j.bbrc.2018.01.185

induction of immune tolerance [3]. Just recently, we identified Gata4 as a master regulator of hepatic microvascular specification [4]. In liver fibrosis and cirrhosis, but also during hepatocarcinogenesis or in liver metastases, LSEC lose their unique morphology and expression pattern by undergoing a process called “capillarization” [5,6].

Through comparative gene expression profiling of LSEC and lung microvascular endothelial cells (LMEC), we identified GPR182 as a potential LSEC-specific molecule [7]. Independent comparative gene expression profiling from mouse liver, lung and brain EC also showed enriched levels for GPR182 mRNA in liver EC [8]. Notably, GPR182 was down-regulated in the livers of our mice with LSEC-specific loss of Gata4 [4].

G protein-coupled receptor (GPR182) 182 is a Class A G protein-coupled receptor (GPCR) that belongs to the subgroup of chemokine receptors [9]. GPCRs are membrane-bound surface molecules that translate external signals into intracellular signaling, and are thereby involved in a multitude of biological processes. Due to their physiological relevance, GPCRs represent promising targets for pharmacological treatment [10]. Notably, up to 40% of all drugs in clinical use exert their functions via GPCRs [11]. The predicted structure of GPR182 is typical of GPCRs featuring an extracellular N terminus, an intracellular C terminus, and seven transmembrane helices [12]. Adrenomedullin (ADM) had been suggested to be a potential ligand for GPR182, however, this hypothesis was not confirmed [13,14]. Thus, GPR182 has remained an orphan GPCR so far. GPR182 expression was found in human heart, skeletal muscle, immune system, adrenal gland and liver using Northern Blot analysis [15]. Furthermore, vascular endothelial cells of zebra fish embryos and developing mouse endothelial cells have been found to express GPR182 [16,17].

Recently, Kechele and colleagues systematically investigated GPR182 expression during development and adulthood using GPR182 reporter mice [18]. In the adult mouse, GPR182 *lacZ* expression was seen in endothelial cells of heart, lung, liver, spleen and lymph node. Moreover, GPR182 was expressed in podocytes of the kidney, spermatocytes and in intestinal stem cells (ISCs) throughout the intestine. In the intestine, GPR182 acts as negative regulator of proliferation during regeneration and adenoma formation by inhibiting ERK signaling.

In human tissues, expression of GPR182 has not yet been comprehensively studied. In addition, the endothelial functions of this orphan GPCR have hitherto remained elusive.

2. Methods

2.1. Patients and controls

Healthy and tumorous tissue samples were retrieved from the Institute of Pathology, University Medical Center Mannheim. All experiments were approved by the institutional ethics board (Medical Ethics Board II, University Medical Center Mannheim, Heidelberg University, Germany; approval 2015-868R-MA). All experiments were carried out in accordance with the Declaration of Helsinki.

Healthy and pathological liver samples (liver fibrosis, liver cirrhosis, HCC) were provided by the tissue bank of the National Center for Tumor Diseases (NCT, Heidelberg, Germany) in accordance with the regulations of the tissue bank and the approval of the ethics committee of Heidelberg University.

2.2. Antibodies, immunohistochemistry, immunofluorescence and microscopy

First antibodies used in this study are listed in Table S1. Formalin-fixed paraffin-embedded (FFPE) human tissues were cut (1-5μm), sections were deparaffinized and rehydrated according to standard protocols. Antigen retrieval (pH6) was performed at 95 °C in a water bath for 1 h. Sections were blocked with 10% BSA, incubated with first antibodies diluted in 0.5% BSA, followed by incubation with appropriate secondary antibodies. Secondary antibodies are listed in Table S2.

Immunoperoxidase specimens were analyzed using Nikon Eclipse Ni microscope and Nikon DS-Ri1 camera (Nikon Instruments, Tokyo, Japan). Immunofluorescent specimens were analyzed using an inverted Leica SP5 Mid Multi-Photon system (Leica Microsystems, Mannheim, Germany). Excitation and detection wave lengths for confocal microscopy were as follows: 488 nm excitation and 518 nm emission maximum for AF488, 543 nm excitation and 570 nm emission maximum for Cy3, 642 nm excitation and 661 nm emission maximum for TO-PRO-3-Iodide. Images were acquired in a sequential mode. Contrast of images was adjusted using Fiji software [19].

2.3. Tissue microarray analysis

Tissue microarrays (TMA) were assembled as previously described [20]. The two tissue microarrays included 126 samples of tumorous and peritumoral regions. Immunoperoxidase staining was carried out as previously described using an anti-GPR182 antibody (HPA027037). Tissue dots were analyzed by light microscopy differentiating between positive and negative staining together with a board-certified pathologist.

2.4. Expression and reporter plasmids

Human and murine GPR182 cDNA was purchased from Sourcebioscience (human GPR182: IRATp970E0545D, Clone ID: 5185932; murine GPR182: IRCKp5014A034Q, Clone ID: 40129794; Cambridge, UK). Human and murine GPR182 genes were cloned into a lentiviral vector (pLADR3) containing a RFP reporter gene. Clones were sequenced without any mistakes by LGC Genomics (Berlin, Germany). The following reporter luciferase plasmids were purchased from Promega: pGL4.29[luc2P/CRE/Hygro], pGL4.30 [luc2P/NFAT-RE/Hygro], pGL4.33[luc2P/SRE/Hygro] Vector, pGL4.34[luc2P/SRF-RE/Hygro] Vector. Plasmids were multiplied using DH5α™ Competent Cells (Thermo Fisher, Waltham, USA) and Qiagen Mini and Maxi Prep Kit (Qiagen, Venlo, Netherlands).

2.5. Cell culture

HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% carbon absorption foetal bovine serum (FCS) and 1% Penicillin and Streptomycin (Merck, Darmstadt, Germany). All cells were grown at 37 °C and in an atmosphere of 5% CO2.

2.6. Cell transfection

HEK293T cells were seeded in 96-well plates and grown for 24 h to approximately 75% confluency. X-tremeGENE™ 9 DNA Transfection Reagent (Roche, Mannheim, Germany) was used for transfection according to the manufacturer's instructions.

2.7. Reporter luciferase assay

Bright-Glo™ Luciferase Assay System (Promega, Madison, USA) and Glo Lysis Buffer (Promega, Madison, USA) were used according to the manufacturer's instructions.

Approximately 30 h (CRE, SRE, SRF-RE) or 48 h (NFAT-RE) post transfection, cells were washed with PBS and incubated with Glo Lysis

Buffer. RFP fluorescence was measured using a microplate reader (Tecan, Männedorf, Switzerland) set to 548 nm excitation, 610 nm emission, 20 μs integration, 25 flashes and gain of 170%. After adding Bright-Glo™ Reagent, Luminescence was measured in a microplate reader (Tecan, Männedorf, Switzerland). All fluorescence and luminescence values were background corrected. Finally, luminescence values were normalized to corresponding RFP fluorescence signal.

For downstream stimulation, 24 h (CRE, SRF-RE, SRE) or 32 h (NFAT-RE) post transfection, cells were washed twice with PBS and culture medium was replaced with stimulation medium. Stimulation media were prepared as follows: 3 μM forskolin (Sigma-Aldrich, Darmstadt, Germany) in 10% FCS for CRE reporter cells, 1 μM Ionomycin (Sigma-Aldrich, Darmstadt, Germany) + 1 ng/ml PMA (Sigma-Aldrich, Darmstadt, Germany) in 10% FCS for NFAT-RE reporter cells, 10 ng/ml PMA in 20% FCS for SRE reporter cells, 20% FCS for SRF-RE reporter cells.

2.8. Statistical analysis

Qualitative values were presented as percentage for categorical variables or means for normally distributed values (luciferase assay). Statistical differences between groups were analyzed by unpaired two-sample *t*-test. To analyze associations between categorical variables Chi-square test was performed. Two-tailed P values below .05 were considered significant. Statistical analysis was carried out using JMP®, Version 11. SAS Institute Inc., Cary, NC, 1989–2007.

3. Results and discussion

3.1. Endothelial GPR182 is restricted to EC with sinusoidal endothelial differentiation

Previous work by us and others reported high GPR182 expression levels in mouse and rat LSEC [7,8,18]. To analyze cell-type-specific expression of GPR182 in human liver sinusoidal endothelial cells, we used healthy samples from human liver for immunoperoxidase and immunofluorescence staining with a commercially available antibody against human GPR182. The specificity of the antibody was confirmed by Western Blotting and immunocytochemistry (ICC) of HEK293T cells transfected with human GPR182 in comparison to empty vector (EV)-transfected HEK293T cells devoid of GPR182 expression (Fig. S1). In healthy human liver tissue, we observed a strictly sinusoidal endothelial expression pattern for GPR182, as analyzed by immunohistochemistry and co-immunofluorescence with LSEC marker CD32b and Kupffer cell marker CD68 (Fig. 1A–F). Regarding zonal endothelial marker expression in the liver, GPR182 expression was predominantly seen in the mid-zonal area. GPR182 was neither expressed in EC of the central vein nor in EC of the hepatic artery and portal vein (Fig. 1A–D).

A broader screening for human organs containing EC with sinusoidal endothelial differentiation revealed a similar expression pattern of GPR182 restricted to sinusoidal EC in human spleen, lymph node and bone marrow (Fig. 2A–J). We confirmed the sinusoidal endothelial expression of GPR182 by co-staining with CD31 (continuous endothelium), Meca79 (high endothelial venules), CD68 (macrophages) and STAB2 (sinusoidal endothelium) (Fig. 2A–J). In contrast, continuous EC in the lungs, heart, kidney and the skeletal muscle did not express GPR182 (Fig. S2).

The sinusoidal endothelial expression pattern demonstrated here for GPR182 is similar to other known LSEC markers such as STAB2 or CD32b [21]. In general, the molecular similarity of liver, spleen and bone marrow EC is well-known [22]. In our analysis, the strongest sinusoidal endothelial expression of GPR182 was found in the spleen as analyzed by immunohistochemistry which is in line with mRNA data from Expression Atlas [23]. Interestingly, recently published data demonstrated that GPR182 knockout mice show a significantly increased splenic weight [18]. Unfortunately, no further investigation of this phenotype was performed by these

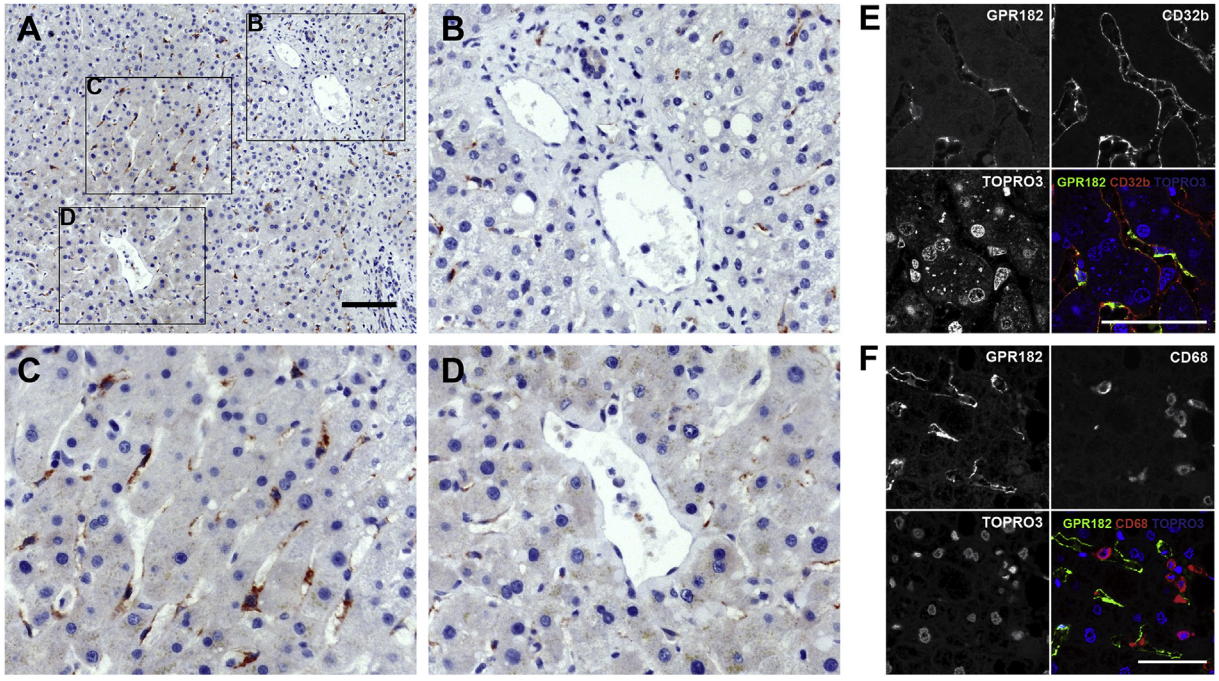

Fig. 1. LSEC express GPR182 in healthy liver tissue.
(A) Representative IHC of GPR182 in healthy human liver (n = 5) and higher magnification of the periportal zone (B), midzonal area (C) and pericentral zone (D). (E) GPR182 co-immunofluorescence (IF) with LSEC marker CD32b and nuclear marker TOTO-3 in healthy human liver (n = 4). (F) GPR182 co-IF with Kupffer cell marker CD68 and nuclear marker TOTO-3 in healthy human liver (n = 3). Scale bars: 100 μm.

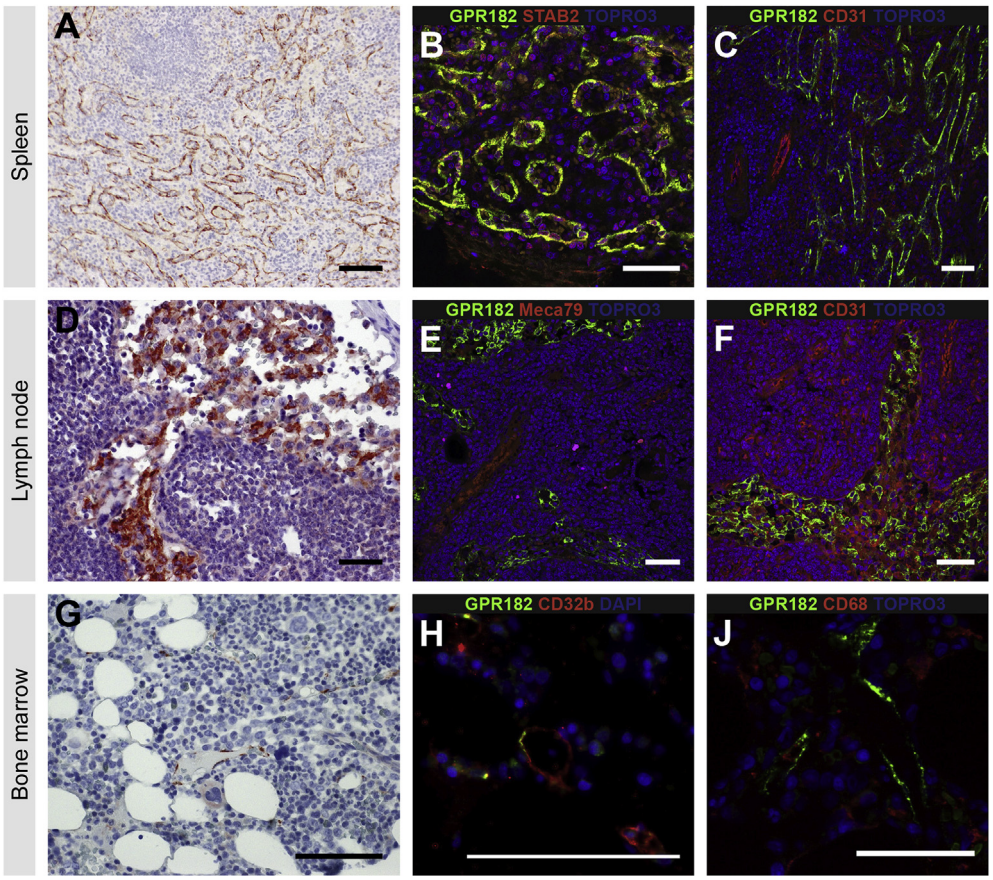

Fig. 2. Sinusoidal endothelial GPR182 expression in human spleen, lymph node and bone marrow.
Representative IHC of GPR182 in healthy human spleen (n = 4) (A), lymph node (n = 14) (D) and bone marrow (n = 3) (G). GPR182 co-IF with sinusoidal endothelial cell markers STAB2 (B) and CD32b (H), high endothelial venules marker Meca79 (E), continuous endothelial cell marker CD31 (C, F), and macrophage marker CD68 (J) in healthy human spleen, lymph node and bone marrow. At least three specimens each were analyzed. Scale bars: 100 μm.

authors. As splenomegaly is typically seen because of ineffective bone marrow hematopoiesis and subsequent extramedullary hematopoiesis in myelofibrosis, it may be hypothesized that endothelial GPR182 might be required for proper hematopoiesis [24].

3.2. Loss of endothelial GPR182 in hepatocellular carcinoma (HCC)

The loss of organ-specific endothelial differentiation in the liver, i.e. sinusoidal capillarization, is involved in metabolic and fibrotic liver diseases and in liver cancer [5,25–27]. Therefore, a human hepatocellular carcinoma (HCC) tissue microarray (TMA) containing 68 tumorous and 58 peritumoral samples was analyzed for GPR182 expression. Immunohistochemistry revealed a significant downregulation of endothelial GPR182 in tumorous versus peritumoral tissues ($p = 0.0105$) (Fig. 3A–E). However, only a minority of peritumoral samples displayed endothelial GPR182 expression (9/58) (Fig. 3E). As HCC most frequently arises in fibrotic and cirrhotic liver tissue [28], we analyzed human tissues of fibrotic and cirrhotic livers for GPR182 expression. As expected, reduced endothelial GPR182 expression was also found in tissues with liver fibrosis and cirrhosis (Fig. 3F–L).

The loss of organ-specific EC marker expression in HCC was previously reported by us. LSEC markers such as Stabilin-2, Lyve1 and CD32b were found to be down-regulated and the continuous EC marker CD31 was upregulated in tumorous tissues indicating that endothelial transdifferentiation is an important event in HCC development and that organotypic endothelial differentiation is required for proper organ function [5]. Interestingly, altered GPCR signaling has been implicated in diseases initiation and progression in various cancers [29]. Peritumoral fibrosis or cirrhosis could be a reason for low GPR182 expression and consequently low number of positive samples in the peritumoral TMA samples [28]. Analysis of fibrotic and cirrhotic liver tissues confirmed this assumption showing loss of GPR182 in both, liver fibrosis and cirrhosis. Recent work by our group identified transcription factor Gata4 as master regulator of organ-specific endothelial differentiation in the liver [4]. Liver endothelial cell-specific deficiency of Gata4 did not only result in embryonic capillarization of the liver sinusoids and fibrosis, but also caused downregulation of GPR182 mRNA in the liver supporting a role of GPR182 in organ-specific EC differentiation.

3.3. GPR182 differentially regulates canonical GPCR signal transduction in vitro

As suggested by Kechele and colleagues, GPR182 is involved in MAPK/ERK-signaling and thereby regulates the proliferative capacity of the intestine. However, other GPR182-dependent downstream signaling pathways have not been characterized yet. To elucidate the involvement of GPR182 in canonical GPCR signaling pathways, we combined ectopic GPR182 overexpression in HEK293T cells with a luciferase reporter assay system. This bioluminescent reporter assays system detects activity of various response elements, namely CRE, NFAT-RE, SRE, and SRF-RE [30]. Thereby, GPR182-dependent activation or inhibition of the four

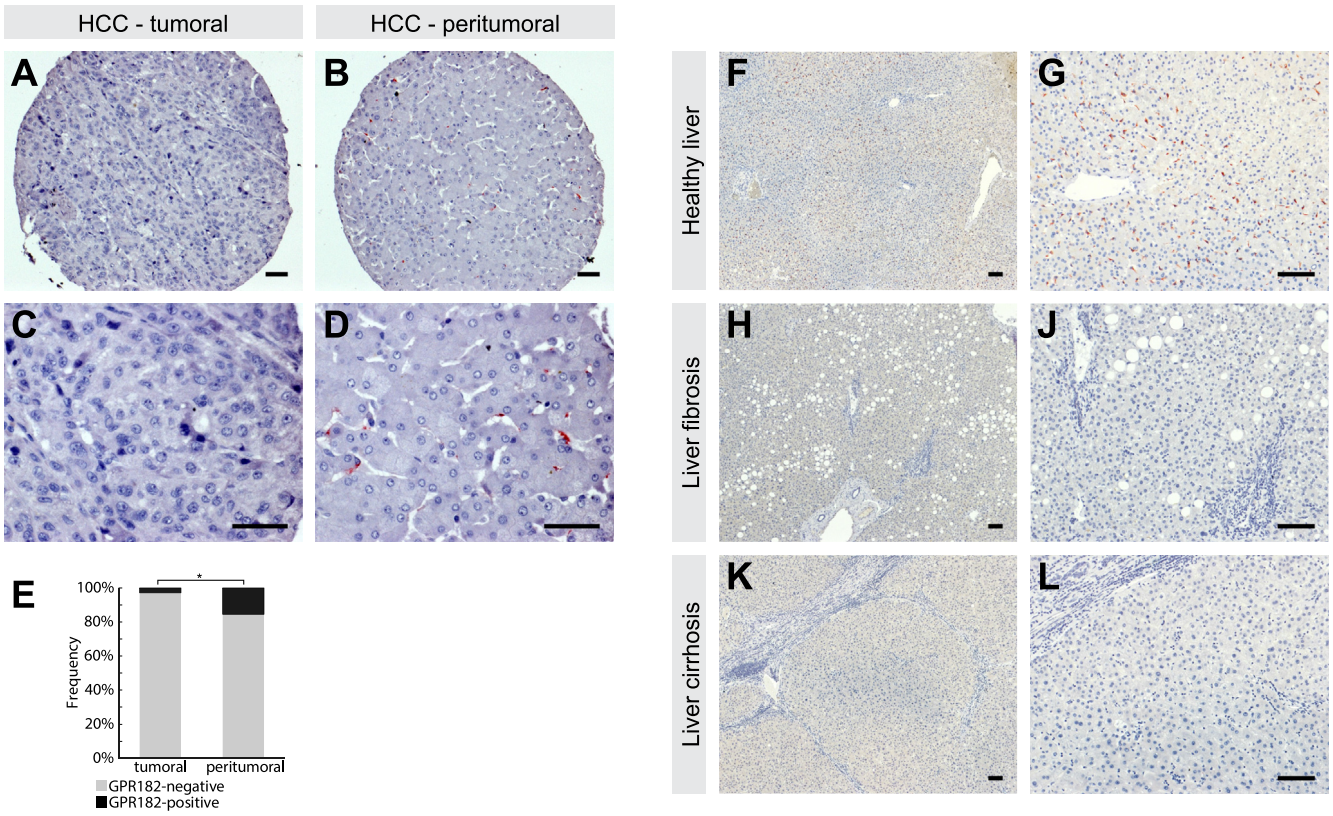

Fig. 3. GPR182 is downregulated in liver fibrosis, liver cirrhosis and HCC on protein level.
(A-D) Representative IHC of GPR182 in tumorous (A, C) and peritumoral HCC samples (B, D) of the tissue microarray used in this study. (E) Statistical analysis of GPR182 expression in tumorous versus peritumoral samples ($p = 0.0105$). IHC of GPR182 in healthy liver (F, G), liver fibrosis ($n = 4$) (H, J) and liver cirrhosis ($n = 5$) (K, L). Scale bars: 100 μm.

GPCR canonical pathways was analyzed: cAMP-RE (CRE) for G protein alpha or cAMP signaling, NFAT-RE for G protein q or Calcium signaling, SRE for G protein i or ERK signaling and SRF-RE for G protein 12 or RhoA signaling.

GPR182-overexpressing HEK293T cells exhibited significantly increased activity for CRE and NFAT-RE reporter luciferases. A 2.9-fold increase of CRE activity was measured ($p = 0.0009$) (Fig. 4A), while the increase of NFAT-RE was 3.5-fold ($p < 0.0001$) (Fig. 4A). In contrast, GPR182 significantly diminished the activity of SRE and SRF-RE reporter luciferases (Fig. 4A). The reporter activity for SRE and SRF-RE was decreased by 79% ($p = 0.0097$) and 78% ($p < 0.0001$), respectively. Thereby, we demonstrate in vitro that GPR182 signals via the classical pathways known for GPCRs. GPR182 overexpression activated cAMP and calcium signaling, whereas ERK and RhoA signaling was inhibited in HEK293T cells without stimulation.

Ligand–independent, "constitutive" signaling is a well-known mechanism of action for GPCRs. Martin and Co-workers demonstrated, for example, that constitutive activity of some GPCRs activates the cAMP signaling pathway, while it inhibits the same pathway upon downstream stimulation using forskolin [31]. We therefore analyzed the effects of GPR182-overexpression upon pathway stimulation downstream of the receptor. The following downstream stimulants were used: forskolin (CRE), PMA and Ionomycin (NFAT-RE), Ionomycin and 20% FCS (SRE), 20% FCS (SRF-RE). GPR182-overexpressing cells revealed significantly less cAMP pathway activity upon forskolin stimulation compared to EV-control ($p = 0.0005$) (Fig. 4B). Thus, GPR182 stimulated cAMP pathway baseline activity while inhibiting the same pathway upon forced downstream stimulation by forskolin. On the contrary, GPR182 had no significant effect upon downstream stimulation of the constitutively activated NFAT pathway ($p = 0.5670$) (Fig. 4B). Moreover, downstream stimulation of the SRE and SRF pathways did not alter GPR182-mediated baseline inhibition of these pathways (Fig. 4B).

Our results corroborate the in vivo findings of Kechele et al. showing that increased ERK activity is found in colon stem cells of GPR182 KO mice in comparison to controls [18]. ERK signaling in endothelial cells promotes arterial and lymphatic EC specification downstream of VEGF [32]. Considering GPR182 as a protein specific for LSEC differentiation, inhibitory effects of GPR182 on ERK-signaling might prevent EC-subtype differentiation into either an arterial or lymphatic phenotype. RhoA signaling is also associated with endothelial differentiation and function regulating vascular hyperpermeability [33].

In addition, Ras/ERK and Rho/actin cascades lead to cell proliferation and differentiation via immediate early genes controlled by transcription factor SRF [34]. SRF acts as regulator in embryonic, adult and pathological angiogenesis by controlling tip cell behavior during sprouting angiogenesis and by regulating genes which are important for cell migration including Myl9 [35]. Regarding organ-specific microvascular endothelial cells, SRF is essential for maintenance of cerebral small vessel integrity. Hence, endothelial SRF knock-out mice suffer from loss of blood-brain-barrier integrity and intracerebral hemorrhage [36]. In the liver, GPR182 may maintain the high permeability of LSEC by inhibiting SRF.

Vascular homeostasis depends on a balanced maintenance of the endothelial barrier. While RhoA signaling is responsible for hyperpermeability, endothelial cAMP signaling is well known to improve barrier function in ECs [37]. In HUVEC, cAMP promotes VE-cadherin dependent EC cell adhesion through Epac-Rap1 signaling and thereby improves EC barrier function [38]. GPR182

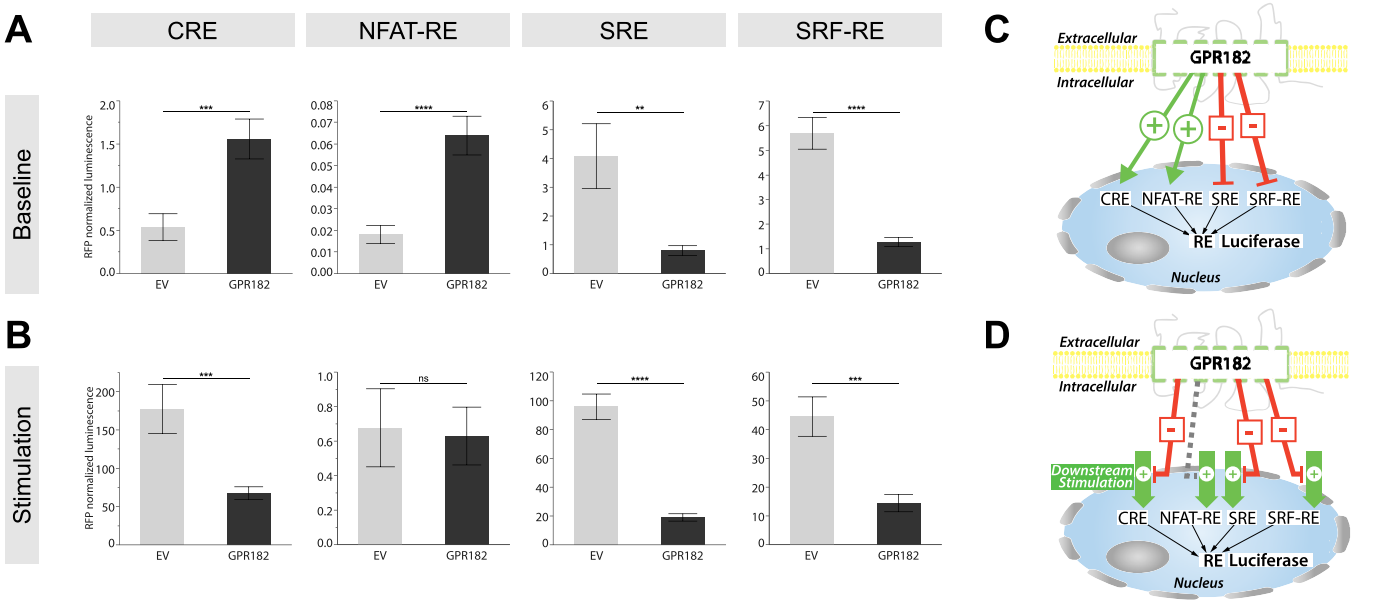

Fig. 4. GPR182-dependent downstream signaling in HEK293T cells in vitro.
(A, C) Reporter luciferase activity in GPR182-versus EV-transfected HEK293T cells. (B, D) Reporter luciferase activity in GPR182-versus EV-transfected HEK293T cells upon downstream stimulation with forskolin (CRE), PMA and Ionomycin (NFAT-RE), Ionomycin and 20% FCS (SRE), and 20% FCS (SRF-RE). Error bars: s.e.m.

overexpression is associated with slightly, but significantly increased cAMP signaling in base line which might improve the homeostatic endothelial barrier. However, the activating effect of GPR182 on the cAMP pathway was inverted upon forced downstream stimulation using forskolin. This phenomenon was described earlier, however its function remains elusive [31]. As cAMP signaling enhances barrier function, one could speculate that GPR182 is capable of inhibiting over-activation of this pathway maintaining high permeability with low level adherence junctions.

In conclusion, this is the first study to comprehensively investigate the endothelial expression of GPR182 in human tissues. GPR182 is a novel marker molecule of sinusoidal endothelial cells in liver, spleen, lymph node, and bone marrow which is lost upon sinusoidal capillarization in liver fibrosis/cirrhosis and liver cancer. Notably, GPR182 is involved in signal transduction using classical GPCR pathways in vitro and may mediate sinusoidal endothelial differentiation (ERK) and hyperpermeability (SRF).

Funding

Supported, in part, by grants from the German Research Foundation (Deutsche Forschungsgemeinschaft) GRK2099/RTG2099, project 7 (to C.G. and S.G.), SFB-TRR209, project C3 (to S.G.).

Conflicts of interest

The authors declare no conflict of interest involving this article.

Acknowledgements

We thank Alexandra Demory, Hiltrud Schönhaber, Jochen Weber, Monica Adrian and Sayran Arif-Said for excellent technical support. We acknowledge the support of the Core Facility Live Cell Imaging Mannheim at the Center for Biomedicine and Medical Technology Mannheim (German Research Foundation grant DFG INST 91027/9-1 FUGG and DFG INST 91027/10-1 FUGG).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2018.01.185.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.01.185.

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