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An immortalized human liver endothelial sinusoidal cell line for the study of the pathobiology of the liver endothelium

Romain Parent [a,b,c], David Durantel [a,b,c], Thomas Lahlali [a,b], Aurélie Sallé [a,b], Marie-Laure Plissonnier [a,b], Daniel DaCosta [a,b], Gaëtan Lesca [a,b,d], Fabien Zoulim [a,b,c,e], Marie-Jeanne Marion [a,b], Birke Bartosch [a,b,c,*]

[a] INSERM U1052, Centre de Recherche en Cancérologie de Lyon, F-69000 Lyon, France
[b] Université de Lyon, F-69000 Lyon, France
[c] DevWeCan Laboratories of Excellence Network (Labex), France
[d] Service de Genetique Moleculaire et Clinique, CHRU Lyon, Hopital Edouard Herriot, Lyon, France
[e] Hospices Civils de Lyon (HCL), Lyon, France

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ABSTRACT

Background: The endothelium lines blood and lymph vessels and protects underlying tissues against external agents such as viruses, bacteria and parasites. Yet, microbes and particularly viruses have developed sophisticated ways to bypass the endothelium in order to gain access to inner organs. De novo infection of the liver parenchyma by many viruses and notably hepatitis viruses, is thought to occur through recruitment of virions on the sinusoidal endothelial surface and subsequent transfer to the epithelium. Furthermore, the liver endothelium undergoes profound changes with age and in inflammation or infection. However, primary human liver sinusoidal endothelial cells (LSECs) are difficult to obtain due to scarcity of liver resections. Relevant derived cell lines are needed in order to analyze in a standardized fashion the transfer of pathogens across the liver endothelium. By lentiviral transduction with hTERT only, we have immortalized human LSECs isolated from a hereditary hemorrhagic telangiectasia (HHT) patient and established the non-transformed cell line TRP3. TRP3 express mesenchymal, endothelial and liver sinusoidal markers. Functional assessment of TRP3 cells demonstrated a high capacity of endocytosis, tube formation and reactivity to immune stimulation. However, TRP3 displayed few fenestrae and expressed C-type lectins intracellularly. All these findings were confirmed in the original primary LSECs from which TRP3 were derived suggesting that these features were already present in the liver donor. We consider TRP3 as a model to investigate the functionality of the liver endothelium in hepatic inflammation in infection.

1. Introduction

Liver sinusoidal endothelial cells (LSECs) form the interface between the microvascular sinusoidal compartment of the liver and the liver parenchyma. LSECs thus form a protective barrier that senses pathogens and has a predominant role in receptor-mediated clearance of infectious agents and toxins from the blood [1]. Among the different types of endothelia, the liver endothelium in particular is thought to serve as an important sink for elimination and degradation of pathogens and digestive products, and also to regulate inflammation, leukocyte recruitment and immune responses [2,3]. However, hepatotropic viruses have developed means to overcome the protective filter formed by LSECs in order to gain access to the underlying liver parenchyma. Indeed, Duck Hepatitis B virus is thought to cross the liver endothelium via transcytosis [4], and a number of other hepatotropic viruses were shown to specifically bind C type lectins expressed on endothelial cells [5–7]. Finally, LSECs are a reservoir for mCMV latency [8]. Thus several viruses can cross the protective liver endothelium in order to gain access to and infect the liver parenchyma. However, the limited access to human liver samples renders the work with primary human LSECs difficult and it remains technically challenging to explore the interaction between LSECs and hepatotropic viruses in more detail. Indeed, with the exception of one approach which describes the reversible immortalization of primary human

* Corresponding author at: Cancer Research Center Lyon, Inserm U1052 – CNRS UMR 5286, Equipe 15, 151 Cours Albert Thomas, 69424 Lyon, Cedex 03, France. Fax: +33 4 72 68 19 70.
E-mail address: Birke.Bartosch@inserm.fr (B. Bartosch).

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LSECs, which was however based on the use of hTERT as well as SV40T [9], no adequate culture systems and non-transformed, tissue culture adopted human LSECs are available, and this lack has hampered the elucidation of the functional relevance of LSECs in the infection process of the liver [2]. In an attempt to develop endothelial cell lines of intrasinusoidal origin for liver physiopathology research, immortalized but untransformed hepatic endothelial foci were isolated from the liver of a hereditary hemorrhagic telangiectasia (HHT) patient. Phenotypic and functional characterization allowed the establishment of the cell line TRP3. These cells are of mesenchymal and endothelial origin, display a rapid endocytic capacity, responsiveness to mediators of inflammation and differentiation, and express a number of liver sinusoidal endothelium-specific markers.

2. Materials and methods

2.1. Patient of origin

The patient from whom TRP3 originate is a 65 year-old female with a history of hepatic arteriovenous malformations related to HHT. Clinical diagnosis of HHT was made according to international consensus criteria [10]. The patient suffered from nosebleed and telangiectasia and displayed an aneurysm of the hepatic artery and shunting from the hepatic artery to hepatic veins on MRI, CT-scan and ultrasound/Doppler study. The patient underwent a liver transplantation at the Lyon University Medical Center. Serum and liver samples were harvested soon after transplantation with informed consent from the patient according to French bioethics laws.

2.2. Liver histology and immunostaining

A minimum of 10 representative tissue blocks were systematically taken from the right and the left lobes of the patient liver and serially sectioned at 0.5 cm intervals. Tissues were formalin-fixed, paraffin-embedded, cut in 5 μm sections, stained with standard hematoxylin–eosin–safranin or immunostained with CD31 and CK8 (Dako) or L-SIGN antibody CD209L (R&D Systems) followed by a biotinylated secondary antibody bound to a streptavidin peroxidase conjugate (Lsab + Kit, Dako). Peroxidase activity was revealed using DAB as a chromogen.

For immunofluorescence cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin/PBS and immunostained with antibodies against vimentin (Immunotech, 1/20), von Willebrand factor (Dako, 1/400), CD34 (clone QBEND10, Novocastra, 1/50), L-SIGN (MAB162, R&D systems, 1/50) followed by detection with alexa-488 conjugated goat anti-mouse (Molecular Probes). Counterstaining was performed with 0.25 μg/ml DAPI (Sigma).

2.3. Mutation screening

Germ line mutation screening was performed on DNA isolated from peripheral blood lymphocytes as previously reported [11,12]. The research for somatic mutations on the second allele was performed by dHPLC on DNA extracted from cultured TRP3 cells, followed by automated sequencing. Quantitative Multiplex PCR of Fluorescent Short Fragments was used to search for a loss of heterozygosity [12].

2.4. Isolation and culturing of endothelial cells and cell lines

Endothelial cells (EC) were isolated from the freshly explanted liver according to a modified version of the protocol described by Daneker et al. [13]. Large abnormal intrahepatic vessels and liver parenchyma were separated and treated separately using the same protocol. HTT is characterized by a very strong capillary enrichment of sinusoids in the liver, and this impedes proper hydrodynamics of the perfusion. Therefore, livers were cut into 3–5 mm cubes and incubated with 0.1% collagenase (Sigma–Aldrich) overnight at 4 °C and 1 h at 37 °C. Cubes were then disaggregated and filtered through a 60 mesh filter. Cell suspensions were centrifuged at 4 °C for 10 min at 1800 rpm and washed 3x with Hepes buffered HBSS. Cells were placed on a density gradient of 18%, 13% and 8% metrizamide and centrifuged for 20 min at 2400 rpm at 20 °C. Cells present between the 13% and 18% layers were collected, centrifuged, washed, resuspended in MCDB131 culture medium (Invitrogen), supplemented with 20% fetal calf serum (Fetalclone I, Hyclone), 10 mmol/L L-glutamine, 250 μg/ml AMPc, 50 μg/ml endothelial cell growth supplement (ECGS, Beckton Dickinson), 1 μg/ml of hydrocortisone and penicillin–streptomycin, seeded in collagen-coated Petri dishes (Beckton Dickinson) and incubated at 37 °C in a 5% CO2 atmosphere. TRP3 cells were selected by successive limited trypsination. Immortalization was achieved by lentiviral transduction with a bicistronic vector encoding hTERT and a puromycin selection marker. Throughout passages 4–11 post-immortalization, the most stably growing puromycin-resistant foci were amplified and screened using endothelial markers.

TRP3 cells were routinely cultured in the endothelial selective medium MCDB131, as mentioned above. HUVEC, Huh7 [14] and HepaRG [15,16] cells were cultured as previously described.

2.5. Flow cytometry and Immunoblotting

Flow cytometry was performed using PBFA (PBS, 5% FCS, 0.2% sodium azide) for surface staining and PBFA containing 0.1% saponin for total cellular staining. Antibodies used were anti-SRB1 (1/200 BD Biosciences #610883) and anti-Stabilin2 (1/200 abcam #ab121893), anti-LDLr (1/25 Calbiochem # LP02), anti-CD81 (1/500 BD Biosciences #JS-81), anti-DC-SIGNR # MAB162 and anti-DC-SIGN # MAB161 (1/25 R&D Systems), anti-LYVE1 (1/50 abcam #ab14917) and anti-CD32B (abcam #ab151497), and anti-mouse/rabbit Alexa Fluor 647 # A21237 (1/200 Invitrogen). Immunoblotting was performed under denaturing conditions using 50 μg cell lysate, the indicated primary antibodies and HRP-conjugated secondary antibodies prior to revelation with in-house made chemiluminescent reagent. Quantification was done using ImageQuant software.

2.6. L-SIGN RT-PCR and Southern blotting

1 or 5 μg of total RNA from TRP3 and HepaRG were harvested using Extract-all reagent (Eurobio) and reverse transcribed using One Step RT-PCR kit (Qiagen). RNA integrity was verified by agarose electrophoresis and 28S and 18S RNA pattern visualization. L-SIGN specific primers (5′-CAGTGGCATCAGACTTTTTC-3′; 5′-CCTGGTAGATCTCCTGCA-3′) were used as previously described [17]. After migration of the 418 base pair amplicons on a 1.5% agarose gel, transfer onto Hybond N+ membrane (GE-Healthcare) and prehybridation, hybridization was performed o/n with a ^{32}P radiolabelled oligoprobe (1.10^6 cpm/ml) using Terminal deoxynucleotide Transferase (Roche). The probe sequence was 5′-CCAG AACCTGACCCAGCTTAAAGCTGCAGT-3′.

2.7. Tube formation and endocytosis assays

TRP3 cells and myofibroblasts (MF) were seeded (3×10^4/well) in 24-well plates coated with 0.3 ml of growth factor-containing Matrigel™ (Becton Dickinson) and incubated at 37 °C with complete MCDB131 medium. Images were taken at ×100 magnification (Nikon TE2000E) at 6 h post-seeding. For endocytic uptake, HepaRG and endothelial cells were incubated with 5 or 15 μg/ml of alexa-labeled acetylated LDL (Life Technologies) for the

indicated time intervals in complete medium. Cells were washed with PBS, fixed for 10 min at rt in 4% paraformaldehyde in PBS, washed and counterstained with Hoechst 33342.

3. Results

3.1. Establishment of an immortalized LSEC cell line

LSECs were isolated from the liver of a 65 year-old female patient liver with a history of hepatic arteriovenous malformations related to HHT [18], who underwent liver transplantation. Macroscopically, the liver displayed at its surface large vascular dilatation, many pinhead telangiectases and some abnormally dilated branches of the hepatic artery. Portal veins with thickened walls were observed in the hilar region but also deep inside the parenchyma (data not shown). Periportal remodeled areas, occasionally fibrotic, were diffusely scattered. Under no circumstance the parenchyma was found cirrhotic. Enlarged portal areas contained clusters of dilated vessels, sometimes abutting directly upon the hepatic parenchyma at the limiting plate (Fig. 1A). The inactive connective tissue of the enlarged portal tracts contained a network of numerous vessels and malformed bile ducts (Fig. 1B). Primary LSECs were isolated from several 3 to 5 mm large biopsy cubes based on a protocol described by Daneker et al. [13]. The resulting primary cell population was cultured in MCDB131 endothelial cell growth supplement (ECGS)-containing medium, purified and hTERT-immortalized.

3.2. Differentiation status of TRP3 cells

To characterize TRP3 cells phenotypically, the expression of liver sinusoidal endothelial markers, including vimentin, CD34, von Willebrand factor, and L-SIGN (CD209L) antigens were studied by immuno-fluorescence and -blotting. Vimentin, von Willebrand factor and CD34 are standard mesenchymal and endothelial cell markers [2]. L-SIGN is a recently characterized C-type lectin homologous to DC-SIGN [19] and its expression is thought to be restricted to lymph nodes and LSEC [2,19]. Fig. 1C shows that TRP3 express vimentin and CD34, indicating their mesenchymal origin, as well as von Willebrand factor in typical cytosolic Weibel–Palade bodies confirming their endothelial phenotype. Staining of the C-type lectins L-SIGN (Fig. 1C) and DC-SIGN (data not shown) demonstrated the sinusoidal phenotype of TRP3 cells. The HepaRG hepatocytic cell line, used as negative control, did not stain positive with any of the antibodies used (data not shown). A unique feature of LSECs is their high capacity to eliminate colloids and soluble macromolecules including alpha-collagen chains and hyaluronan. Expression of the hyaluronan receptors LYVE-1 and stabilin 2 as well as of the CD32B antigen has been shown to be a particular feature of LSECs [2] and to disappear upon transdifferentiation in vitro [20]. Expression of stabilin-2, L-SIGN and CD32B was detected by immunoblotting in TRP3 and primary LSECs to similar extents but not in HepaRG cells. Quantification ascertained these data (Fig. 1D). This finding was confirmed by FACS for CD32B (data not shown). Moreover, FACS surface-staining confirmed the presence of LYVE1 not only in primary LSECs but also in TRP3 (Fig. 1E), suggesting that TRP3 are maintaining expression of LSEC specific markers at the surface. However, in contrast to LYVE1, the major pool of L-SIGN and DC-SIGN was found to remain intracellular in TRP3, as determined by FACS staining in the presence and absence of detergent (Supplementary Fig. 1). In order to exclude expression of C-type lectin isoforms with an altered expression pattern as explanation for this finding, expression of the full-length L-SIGN transcript was confirmed by RT-PCR/South-

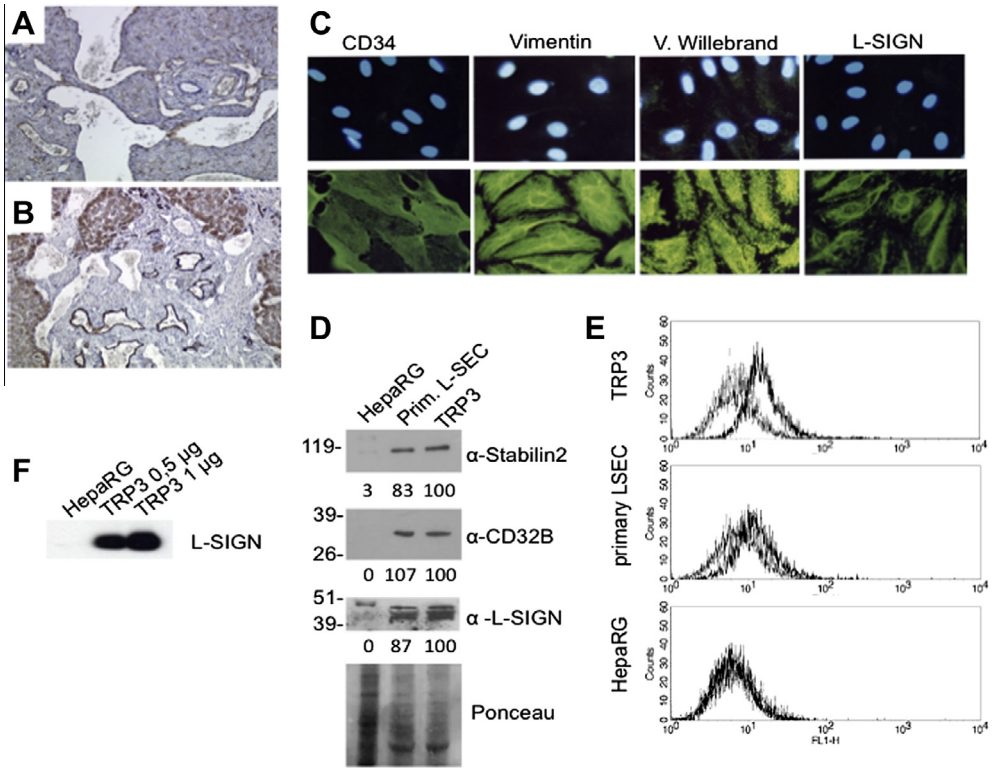

Fig. 1. Histological staining of sections derived from the liver used for isolation of liver sinusoidal endothelial cells and phenotypic characterization of TRP3 cells. (A) CD31 expression in highly remodeled portal tracts. (B) CK8 expression showing the intricacy of numerous vessels and malformative bile ducts. (C) Immunofluorescence of TRP3 cells with the indicated antibodies (right hand panels) or DAPI staining (left hand panels). (D) Immunoblots of total cell lysates of HepaRG, TRP3 and primary LSECs using the indicated antibodies. Quantification of each signal is shown (E) FACS surface staining of HepaRG, TRP3 and primary LSECs using an anti-LYVE1 antibody (black, bold line). Background staining was performed without antibody (dotted line) or with secondary antibody only (black, fine line). (F) Southern blot of HepaRG (5 µg) and TRP3 (0.5 and 1 µg) cDNA with an L-SIGN specific probe.

ern blotting with L-SIGN specific oligos in TRP3 and primary LSECs (Fig. 1F). Moreover, the intracellular localization of both L- and DC-SIGN with concomitant absence from the cell surface was also observed in primary human LSECs (Supplementary Fig. 1) and human umbilical vein endothelial cells (HUVEC; data not shown). In order to ask whether stimulation by inflammation-related cytokines may induce translocation of C-type lectins to the cell surface, TRP3 cells were treated with LPS, IL4 and TNFα. However, these cytokines altered neither the expression pattern of the intracellular DC- and L-SIGN pools nor the total expression levels of these two C-type lectins (data not shown).

To assess the physiological differentiation status of TRP3 cells, their capacity for endocytic uptake was investigated using acetylated low-density lipoprotein (AcLDL). AcLDL is a unspecific ligand that is not only taken up by LSECs but also by dendritic cells which are absent from TRP3 cells pools, but uptake is faster and occurs at lower ligand concentrations in LSECs compared to other cell types [2,21]. Confluent TRP3, primary LSECs and HepaRG were exposed to 5 μg/ml of alexa-conjugated AcLDL, and uptake was followed over time. Fluorescent vesicle formation was observed in TRP3 and primary LSECs after 30 min and increased over time (Fig. 2A). AcLDL uptake was quantified by counting fluorescent foci per cell, and was more rapid in primary LSECs with up to 3-fold more fluorescent punctae at early time points compared to TRP3, but reached similar levels in both cell lines at 120 min (Fig. 2B). No uptake was observed in hepatocytic HepaRG cells under these conditions. Identical results were obtained in a 240 min-long kinetic approach using 15 μg/ml of acLDL (not shown). While these data show that TRP3 have retained the capacity for rapid endocytic uptake at low ligand concentrations, EM analysis of confluent TRP3 cultures demonstrated absence of fenestrae (data not shown), as observed in long-term LSEC cultures.

Tube formation is another hallmark of differentiated endothelial cells. Upon culture on growth factor-reduced collagen matrix, TRP3 cells formed tube-like structures, while myofibroblasts, used as a negative non epithelial/mesenchymal control, did not (Fig. 3A). To test reactivity to inflammatory stimuli, TRP3 cells were exposed to 1 ng/ml LPS. Six hours after treatment, TRP3 cells adopted the expected stress-related, elongated shape, which persisted for three days (Fig. 3B). Morphological changes were furthermore associated with the rapid transcriptional induction of endothelial adhesion molecules (Fig. 3C). TNFα stimulation induced within a few hours the elevation of ICAM1, VCAM1 and E-selectin mRNAs, which returned to baseline after 24 h. Induction of CLEVER-1, which mediates transmigration of leukocytes, occurred between 24 and 48 h after TNFα stimulation.

The molecular cause of HHT is a dysruption of the TGF-β signaling pathway, which can affect endothelial cell differentiation. In 85% of HTT cases mutations in TGF-β receptors have been observed. Therefore, the TGF-β signaling status was assessed in TRP3 cells. Indeed, the cells displayed a p.Ile485Phe missense mutation in the activin receptor like kinase A (ALK1) gene (data not shown), which is known to downregulate endoglin and Alk1 expression [22]. In comparison to HUVEC, TRP3 cells displayed

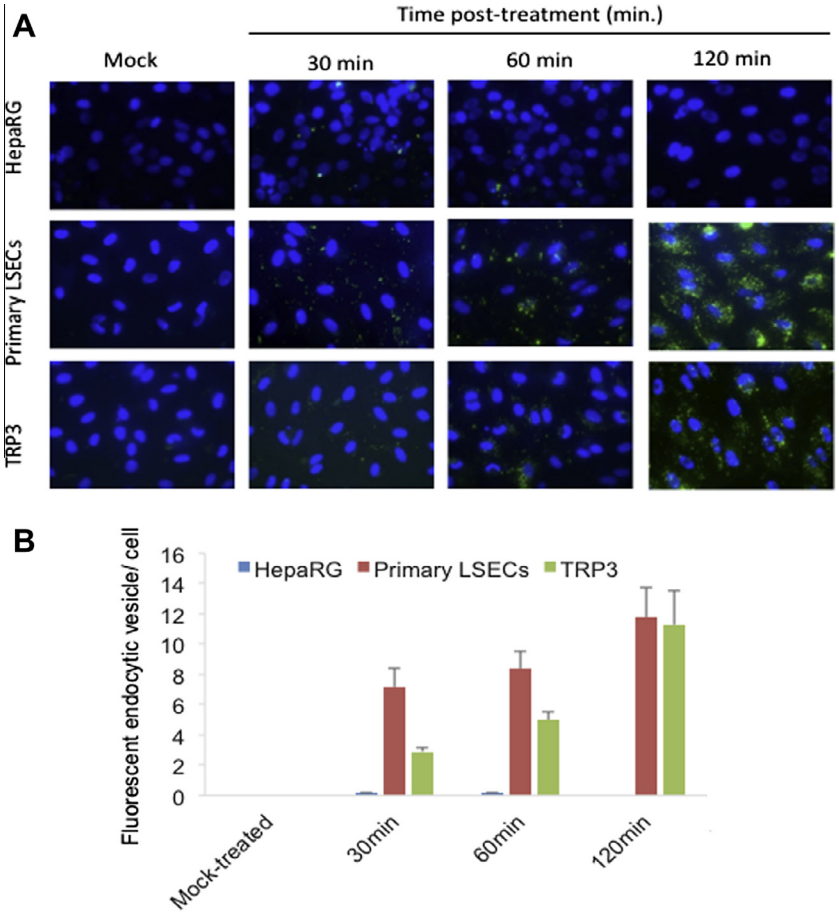

Fig. 2. Endocytosis of acetylated LDL. (A) HepaRG and endothelial cells were incubated with 5 μg/ml of alexa-conjugated acetylated human LDL for the indicated time points in complete medium. After medium removal, cells were fixed, counterstained, mounted and finally visualized by epifluorescence on a Nikon TE2000E microscope equipped with a digital camera. (B) The number of endocytic events per cell (deemed proportional to the number of green fluorescent punctae) was determined using the open access ImageJ NIH software. Results are representative of two experiments.

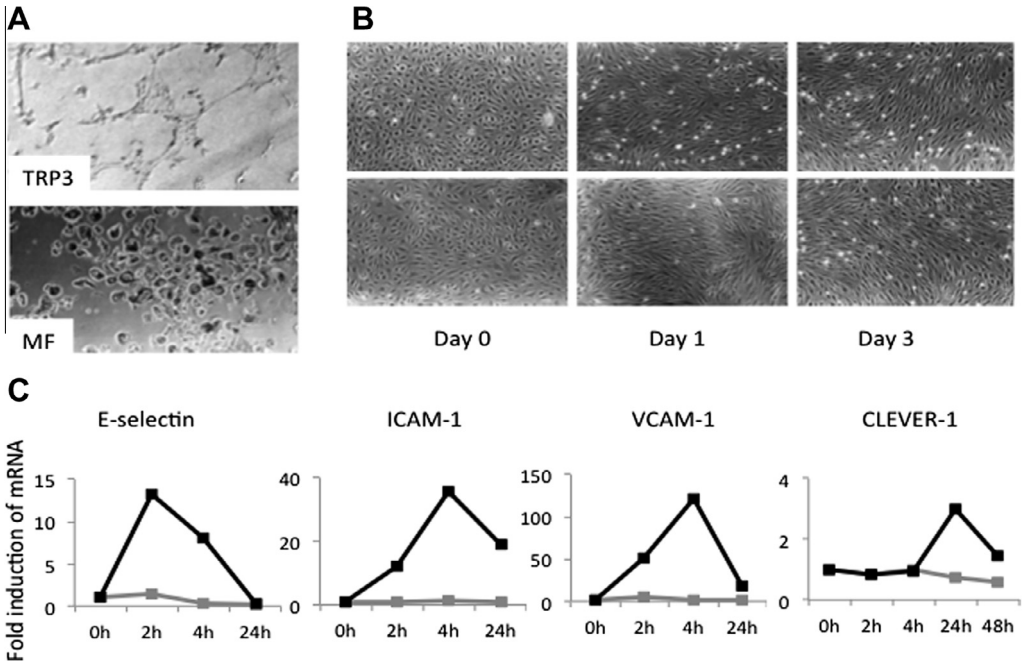

Fig. 3. Differentiation status of TRP3 cells. (A) Tube formation assay on a collagen matrix with myofibroblasts (MF) and TRP3 cells (TRP3). (B) Response of TRP3 to 1 ng/ml LPS in terms of morphology and (C) induction of mRNA levels of adhesion molecules in response to 10 ng/ml TNFα (black lines) or mock treatment (gray lines) (E-Selectin, ICAM-1 $n = 3$; CLEVER-1 $n = 2$).

relative lower levels of endoglin and Alk1 in immunofluorescence and on quantitative Western blots (approx. −30% and 40%; Fig. 4A and B, respectively). To assess functionality of TGF-β signaling in TRP3 cells and sensitivity to activation and migration by hormones of the TGF-β superfamily, the responsiveness of TRP3 cells to bone morphogenetic protein 9 (BMP9), an endothelial differentiation-inducing factor and ligand of ALK1, that induces SMAD phosphorylation and translocation [23] was measured. A 45 min exposure to BMP9 induced Smad-1 phosphorylation (Fig. 4C), suggesting that the ALK1 p.Ile485Phe missense mutation does not impede TGF-β signal transduction in TRP3 cells.

4. Discussion

The liver is composed of parenchymal, i.e. hepatocytic, cells that amount to around 80% of the total liver mass. The rest of liver cells is composed of non parenchymal cells, among which endothelial cells are the most numerous. Although functional links between parenchymal and non parenchymal cells only take place indirectly since both cell types are separated by the space of Disse, endothelio-parenchymal cross-talks do play roles in intrahepatic inflammation and viral infections [2,24]. However, most studies conducted so far that have tried to investigate the interplay of liver resident cell types in infection and inflammation have been hampered by the lack of availability of endothelial partners for hepatocytic cocultures or artificial liver-like microenvironment reconstitution in vitro. In this context, the aim of this study was to develop an immortalized cell line of endothelial origin, that retains the functional characteristics of its tissue of origin. We have shown that TRP3 cells, derived from the explanted liver of an HHT patient, maintained expression a number of typical markers including vimentin, CD34, CD32B, the lectin L-SIGN as well as hyaluronan receptors stabilin-2 and LYVE-1, which are known to be constitutively expressed on LSECs and absent on other hepatic cells and conventional endothelium [2]. In addition, TRP3 cells displayed functional activities that are known to be specific to LSECs. TRP3 cells were capable of rapid endocytic uptake of AcLDL at low ligand concentrations. While AcLDL is not a specific ligand for

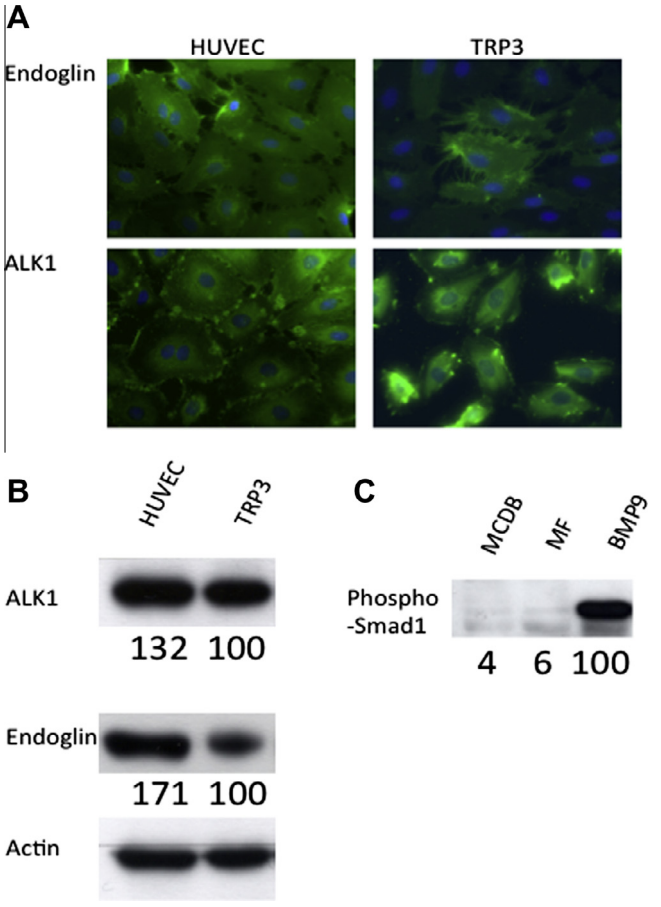

Fig. 4. TGF-β signaling in TRP3 cells. (A) Expression of ALK1 and endoglin by immunofluorescent staining of HUVEC and TRP3 cells (100X). (B) Immunoblot of HUVEC and TRP3 cell lysates with the indicated antibodies. Quantification of each signal is shown (C) TRP3 cells are responsive to the proliferation and migration inhibitor BMP9. Expression of Phospho-Smad-1 45 min after exposure to basal MCDB131, conditioned medium of MFs or 5 ng/ml BMP9. Quantification of each signal is shown.

LSECs, the rapidity of the uptake, in combination with the presence of LSEC specific marker molecules argues very strongly that TRP3 display indeed LSEC-like endocytic properties. Furthermore, TRP3 were responsive to LPS, TNFα and BMP9 and formed vascular tubules, which again is representative of mature endothelial cells in general [2].

L-SIGN, a marker for hepatic sinusoidal endothelial cells but not hepatocytes [19], was expressed at robust levels in TRP3 cells, but was undetectable at the plasma membrane in basal culture conditions. Importantly, L-SIGN was undetectable at the plasma membrane not only in TRP3 but also in isolated primary LSEC and HUVEC, suggesting that intracellular localization of L-SIGN is a more general feature than thought. This issue warrants further study in the future, particularly as so far only one single study has suggested that L-SIGN is expressed at the cell surface [2].

In conclusion, these results demonstrate the endothelial and intrahepatic sinusoidal phenotype of TRP3 cells. They also confirm the physiological responsiveness of TRP3 cells to coagulation and inflammation-related stimuli, as well as their spontaneous ability to endocytose and to organize into vascular tubules. Treatment of TRP3 with neither LPS, IL4 nor TNFα induced the translocation of the predominantly intracellular C-type lectins to the cell surface. Importantly, C-type lectins were also found to be predominantly intracellular in primary human LSECs and HUVECs, suggesting that these capture receptors are absent or only weakly expressed at the cell surface and probably translocate in response to inflammatory stimuli other than those tested here. Hence the TRP3 cell line may offer novel opportunities to hepatology studies involving functional cross-talks between the sinusoidal and parenchymal compartments in inflammatory or infectious settings.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.038.

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