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SOX18, RELA and FAK form a common feed-back circuit enabling lymph endothelial barrier disintegration upon 12(S)-HETE induction

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Abstract

The metabolite 12(S)-HETE is released by cancer cells and destabilizes the endothelial barrier in order to access the lymphatic and blood system. SOX18 is a transcription factor responsible for differentiation and determination of the endothelial lineage and was recently identified as key player in lymph endothelial cell (LEC) retraction. Since the focal adhesion kinase (FAK) is deregulated in many cancer types and was linked to cancer invasion, we investigated whether FAK resides in the same pathway as SOX18 by testing activation upon 12(S)-HETE induction. LECs were transiently transfected with small interfering RNAs (siRNAs) to investigate signaling on the protein and mRNA level. A novel signaling network was discovered which serves as switch for rapidly turning FAK signaling on or off. Furthermore, we found that RELA participated in the here described FAK-SOX18 signaling loop. Since siRNAs are not available for treatment, we tested two natural occurring NF-κB inhibitors curcumin and parthenolide and a clinical trial phase II FAK inhibitor, defactinib, regarding their anti-intravasative effects on lymph endothelial barrier breaching. With the validated “Circular chemorepellent defects” (CIDD) assay a 3D environment was created through growing cancer cells as spheroids which then are placed on a LEC monolayer and co-incubated for 4 hours. Certain drug-combinations additively reduced CCIDs, which formed beneath cancer spheroids by LECs moving away from the cancer-generated stimulus. Thus RELA, FAK and SOX18 are critical compounds for LEC retraction, and therefore facilitate tumor intravasation.

Introduction

Cancer cells moving through the endothelium of the blood or lymphatic system is a crucial step in cancer metastasis. This process is called intra- and extravasation and is initiated by cancer cells releasing molecules that interfere with cellular processes of adjacent vessel walls. 12(S)-HETE is such a metabolite and is produced by lipoxygenases ALOX12 and ALOX15, and by cytochrome-P450-1A1 (CYP1A1). In 1994, 12(S)-HETE was found to be expressed by cancer cells for the first time inducing a mobile mesenchymal phenotype in lymph endothelial cells (LECs) (Honn et al., 1994). Via its two receptors, the high affinity receptor 12-HETER and low affinity receptor BLT2,12(S)-HETE activates mobility proteins such as MLC2 through Ca^{2+}, RHO and MYLK pathways which causes the retraction of endothelial cells (ECs) (Nguyen et al., 2016; Stadler et al., 2017). Recently, SOX18 together with PROX1 were identified to be key players in lymph endothelial cell (LEC) retraction when activated with 12(S)-HETE (Fristiohady et al., 2018). SOX18 belongs to the SOXF-family and is a crucial transcription factor determining endothelial differentiation in embryonic development. The lymphatic system develops from blood endothelial cells overexpressing SOX18 which results in expression of PROX1, a transcription factor uniquely expressed in lymph endothelial cells (François et al., 2008). The most important task of the lymphatic system regarding disease is handling immune response upon pathogen attack and providing information about cancer progression (Oliver, 2004).

The focal adhesion kinase (FAK) is a non-receptor tyrosine kinase localized at focal contacts maintaining turnover and signal transduction of extracellular signals by interacting with integrins and growth factors. These two functions are crucial for cell motility (Mitra et al., 2005). SOX18 and FAK are both factors involved in processes such as differentiation, survival, migration and invasion, occupying a central position in signal transduction, and are therefore often deregulated in many cancers (Olbromski et al., 2018; Sulzmaier et al., 2014). Contribution of FAK activity to LEC retraction is known, but was never tested upon 12(S)-HETE stimulation (Hong et al., 2018). By using siRNAs, we studied the dependence of FAK phosphorylation to SOX18 and vice versa. Additionally, we

investigated the involvement of NF-κB signaling by targeting the transcription factor RELA. RELA occurs predominantly in a heterodimer with p50, which together translocate into the nucleus upon activation. It is a critical compound of the canonical pathway controlling inflammation. In cancer NF-κB is constitutively activated, boosting inflammatory processes (Lawrence, 2009). NF-κB was reported to negatively regulate SOX18 expression in HUVECs, whereas in LECs SOX18 is regulated positively. (Basílio et al., 2013), (Fristiohady et al., 2018). The connection of NF-κB to FAK is also ambivalent since reciprocal activation was reported (Liu et al., 2008; Murphy et al., 2019; Rudelius et al., 2018; Wang et al., 2012).

Since FAK and NF-κB are central proteins in the here discovered signaling loops, we were interested in their abilities as intravasative protagonists. With the “circular chemorepellent induced defects” (CCID) assay we tested pharmacological relevant inhibitors regarding their anti-migratory effects. The CCID assay mimics the natural process of cancer cell intravasation in the body by using 3D-cultured HCT116 colon cancer cell spheroids. Spheroids are placed on a pre-stained LEC monolayer and co-incubated over 4 h. Interaction of tumor cells with the lymph endothelial layer results in formation of a gap in the monolayer beneath the spheroid by migration of LECs away from the stimulus generated by the tumor cells (Fig1A).

Different substances such as inhibitors or natural extracts and even signaling molecules can be tested to measure their anti-intravasative properties based on the extent of LEC retraction. The size of CCIDs give rise to the pro-intravasative activity of the tumor embolus. The smaller the CCIDs, the more effective is the inhibitory substance or gene alteration in preventing cell migration (Fig.1B) (Kerjaschki et al., 2011).

Here, we demonstrate a complex signaling network that unites three major players involved in cancer progression, which are interactively regulating LEC migration upon 12(S)-HETE stimulation.

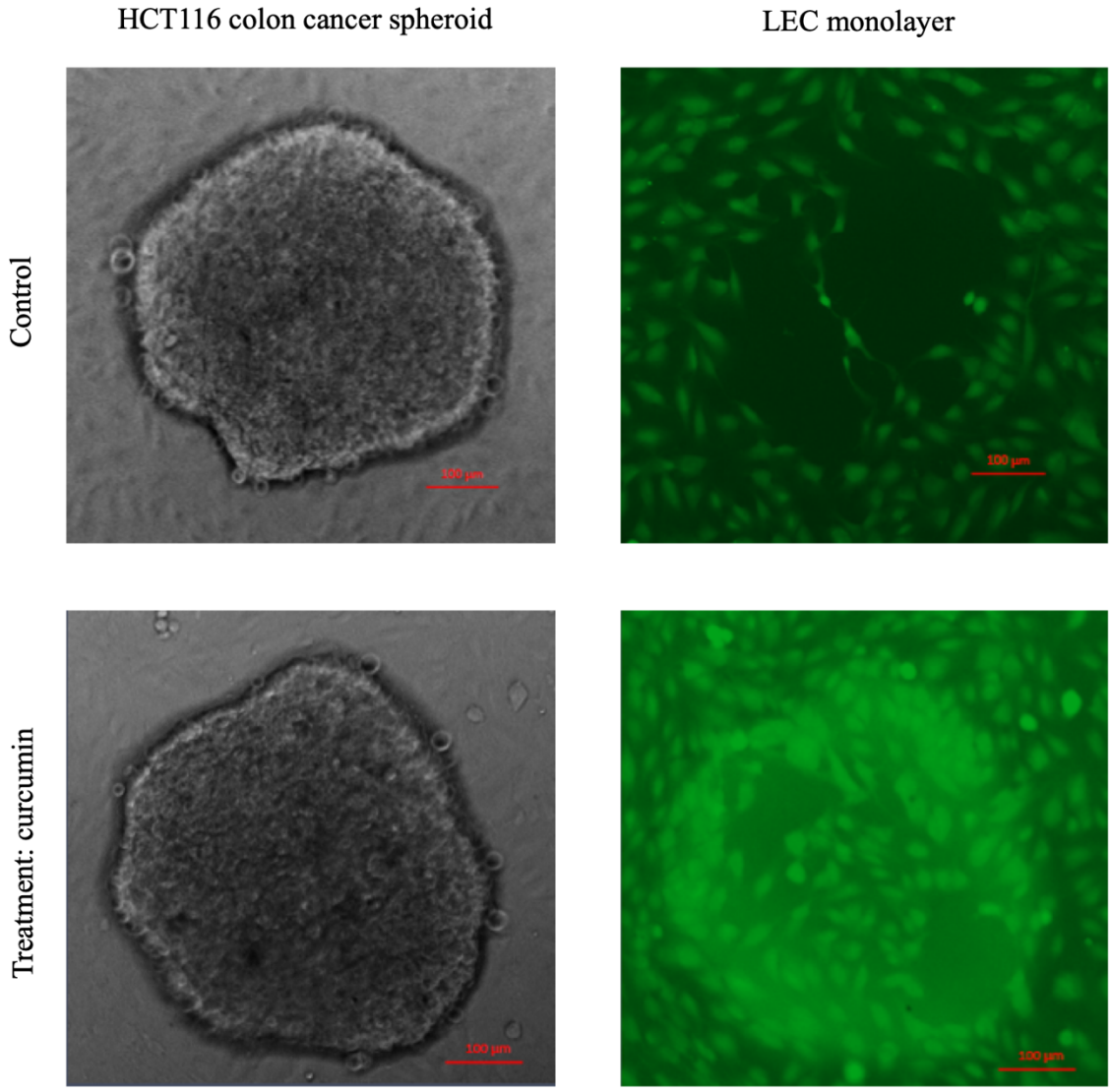

Fig.1: CCID formation in lymph endothelial cell (LEC) monolayer due to cancer cell interaction. (A) HCT116 colon cancer spheroid placed on a LEC monolayer (left). CCID formation in CellTracker Green CMFDA stained LEC monolayer (untreated condition= Control). (B) HCT116 colon cancer spheroid placed on a LEC monolayer (left). Reduced CCID formation in CellTracker Green CMFDA stained LEC monolayer treated with 2µM curcumin showing auto-fluorescence (right). Pictures were taken by using an Axiovert microscope and Zen Little 2012 software. Scale is equivalent to 100µm and indicated as red line in the lower right-hand corner of each picture.

Materials & Methods

Antibodies and Reagents

Rabbit polyclonal antibodies against focal adhesion kinase (FAK, #: 3285; used 1:1000) and phospho-Tyr397-FAK (pFAK, #: 3283; used 1:1000), and mouse monoclonal anti-v-Rel avian reticuloendotheliosis viral oncogene homolog A antibody (RELA/p65, clone L8F6, #: 6956; used 1:1000) were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Rabbit polyclonal anti-SRY-related HMG-box 18 (SOX18, TA324592; used 1:600) was ordered from OriGene Technologies (Rockville, MD, USA). HRP-conjugated swine anti-rabbit antibody (#: P0217; used 1:5000) and HRP-conjugated rabbit anti-mouse (#: P0260; used 1:10000) were from Dako Cytomation (Glostrup, Denmark).

siRNAs against RELA (siRELA; ID: s11914), PTK-2 (siFAK; ID: s11485) and the Silencer Select Negative Control Silencer No. 1 si-RNA (n.t.Co; ID: 4390843) were purchased from Ambion (Life Technologies, Carlsbad, CA, USA),and SOX18 (siSOX18; L-019035-00-0005) from Dharmacon (Lafayette, CO, USA).

qPCR primers for SOX18 (Hs00746079_s1), RELA (Hs00153294_m1), ICAM-1 (Hs00164932_m1), PROX1 (Hs00896294_m1) and ß-actin (Hs01060665_g1) were from TaqMan (Applied Biosystems, Vienna, Austria).

12(S)-HETE (CAS: 54397-83-0) was purchased from Enzo Life Sciences (#: BML-H012-0050, New York, NY, USA), Bay11-7082 (#: 196870) from EMD Millipore Corp. affiliate of Merck, Darmstadt, USA (Bedford, MA, USA). Parthenolide (#: P0667), Arachidonic acid (#: 10931), proadifen hydrochloride (#: P1061), guanfacine (#: G1041), vinpocetine (#: V6382) and curcumin (#: 08511) were from Sigma (Munich, Germany), and defactinib (#: S7654; VS-6063, PF-04554878) from Selleckchem (Houston, TX, USA).

Cell lines

HCT 116 colon cancer cells were purchased from ECACC (Catalogue Nr. 91091005) and cultured in MEM medium supplemented with 10% foetal calf serum (FCS), 1% penicillin/streptomycin (PS) and 1% non-essential amino acids (Gibco/Invitrogen, Karlsruhe, Germany).
Human micro-vessel endothelial cells were purchased from Clonetics™ (Lonza Group, Ltd., Basel, Switzerland). A stable cell line of telomerase immortalized dermal lymph endothelial cells (LECs) was created by Schoppmann et al., 2004 and provided to the department. LECs were grown in EGM-2MV (EBM2-based medium CC3156 & supplement CC4147; Lonza, Basel, Switzerland) and cultivated at 37˚C in a humidified atmosphere containing 5% CO_2.

siRNA transfection

LECs were seeded in 6-well plates and grown to 80% confluence. 1.75 µg of each siRNA was mixed with 15 µl Hiperfect transfection reagent (Cat. no.: 301705; Qiagen GmbH, Hilden, Germany) in 100 µl serum free medium and left at room temperature (RT) for 30 min to allow formation of transfection complexes. Old medium was exchanged for 1.4 ml pre-warmed serum free medium and transfection-mix was added dropwise to the cells and incubated overnight. On the next day the medium was exchanged for serum containing medium and cells were allowed to recover for 24 h.
For CCID assay LECs were seeded in 24-well plates and grown to 80% confluence. Subsequently, the medium was changed to serum free medium and the transfection mix (0.75 µg siRNA, 6 µl Hiperfect transfection reagent, 100 µl serum free medium) was added to the cells and experiments were processed as outlined above.

12(S)-HETE stimulation

Transfected LECs, which were allowed to recover overnight, were starved for 2,5h in serum free medium and stimulated with 1µM 12(S)-HETE for 45 min. Gene and protein expression was analyzed by qPCR and Western blotting, respectively.

SDS-PAGE and Western blotting

LECs were lysed in 2x SDS lysis buffer containing 0.5 M Tris-HCl (pH 6.8), 20% SDS, 10% glycerol, 0.5 M EDTA, phosphatase inhibitor cocktail and protease inhibitor cocktail and sonicated in a pulsed manner with a Sonifier 2000. After centrifugation supernatant was mixed with 6x loading dye and heated for 5 min at 95°C. Equal amounts of protein were separated by SDS-PAGE (80 V for 10 minutes, 110V for 2 h, constant) using Mini PROTEAN Tetracell (Bio-Rad, Hercules, California, USA) following the Bio-Rad General protocol for Western blotting. Subsequently, proteins were electro-blotted (20 V constant, on ice, overnight;) onto Immobilon FL PVDF membrane (0.45 µm pore size; Millipore, Bedford, MA, USA) using transfer buffer containing 20 mM Tris-base, 150 mM glycine, 20% (v/v) methanol, pH 8.5. Membranes were stained with Ponceau S (Sigma-Aldrich, Munich, Germany) to check transfer efficiency and equal loading. Membranes were blocked in 5% dried skimmed milk in TBS-T (1x Tris buffered saline/0,1% Tween 20, pH 7.6) for 1 h and incubated with either anti-pFAK, anti-FAK, anti-RELA and anti-SOX18 antibodies, agitated at 4°C, overnight. Then, membranes were washed three times in TBS-T and incubated with HRP-conjugated swine anti-rabbit antibody, or rabbit anti-mouse antibody at room temperature for 1h. Chemiluminescence was measured with a F1 Lumi-Imager Workstation (Roche, Basel, Switzerland) and densitometry was measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA) and Excel 2013. For repetitive analyses, membranes were stripped in between antibody incubations with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA) at 37° C for 5-10 min followed by 3 washes in TBST-T.

Real time RT-qPCR

RNA extraction of transfected and stimulated LECs was done with the RNeasy Mini Kit 50 and QIAshredder 50 (QIAGEN, Hamburg, Germany). RNA concentration was measured with a NanoDrop Fluorospectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). 2µg RNA were reverse-transcribed using RNA-to-cDNA-EcoDry™ Premix Protocol-At-A-Glance (Takara Bio Europe, Saint-Germin-en-Laye, France). For gene expression analysis TaqMan Gene Expression Master Mix (Applied Biosystems, Vienna, Austria) and TaqMan primers were used and calculated with the Biorad CFX 96 Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA) mRNA levels were calculated using the ΔΔCT method (Livak and Schmittgen 2001).

12(S)-HETE assay

HCT116 cells were seeded in 6-well plates and grown in complete MEM medium to 90% confluence and starved overnight. On the next day cells were treated with 10 µM arachidonic acid and simultaneously with 40 µM proadifen, 40 µM guanfacine, 40 µM vinpocetine or solvent (DMSO) in serum free medium at 37°C for 4 h. Butylated hydroxytoulene (0,001% final concentration; #: B1378, Sigma, Munich, Germany) was added to the collected supernatant (1.5ml) in order to stabilize the secreted eicosanoids, and centrifuged at 2,000 rpm at 4°C for 5 min. Samples were flash frozen in liquid nitrogen and stored at -80°C until further analysis. 12(S)-HETE in the cellular supernatant was extracted by Walter Jäger and measured by Stefan Brenner with minor modifications as described previously (Teichmann et al., 2014) using the 12(S)-HETE enzyme immunoassay kit (EIA, #ADI-900-050; Enzo Life Sciences, Lausen, Switzerland). In detail, supernatant was passed through extraction cartridges (Oasis™ HLB 1 cc, Waters, Milford, MA; equilibrated with 2 × 1 ml methanol, 2 × 1 ml ddH2O immediately before use) followed by washing of cartridges with 3 × 1 ml distilled H2O. Bound 12(S)-HETE was eluted with 500 µl methanol. After the evaporation of methanol with a speed-vac concentrator, the samples were reconstituted with 100 µl assay buffer of the 12(S)-HETE enzyme immunoassay kit (EIA, #: ADI-900-050; Enzo Life Sciences, Lausen, Switzerland) and subjected to 12(S)-HETE

analysis according to the manufacturer´s instructions. Absorbance was measured with a Wallac 1420 Victor 2 multilabel plate reader (Perkin Elmer Life and Analytical Sciences Waltham, MA, USA). The concentration of 12(S)-HETE in the cellular supernatant was normalized to cell number to account for differences in the cell number.

Spheroid formation

Per spheroid 6000 cells of the cell line HCT116 were added to complete MEM medium containing 20% methyl cellulose solution (final conc. 0.3%) and seeded into U-bottom shaped 96 well plates (Cellstar, #: 650185, Greiner bio-one, Kremsmünster, Oberösterreich, Austria). After centrifugation at 1500 rpm for 15 min, spheroids were grown at 37°C and 5% CO2 for 2 days.

CCID assay

LECs were seeded in 24-well plates (Costar #: 3524, Sigma-Aldrich, Munich, Germany) and grown to almost a 100% confluence. To be able to measure the size of the cell free areas (circular chemorepellent-induced defects; CCIDs), which are formed in the endothelial monolayer directly underneath the tumor spheroids, LECs were stained with CellTracker™ Green CMFDA purchased from Life Technologies (Carlsbad, CA, USA) at 37°C for 1 h. For treatment with curcumin and defactinib HCT116 spheroids as well as the LEC monolayers were washed with PBS and pre-incubated with different concentrations of these compounds at 37°C for 20 min. For parthenolide, only the spheroids were pre-incubated at 37°C for 20 min. For combination experiments LEC monolayer was pre-incubated with Bay11-7082 for 15min and subsequently with defactinib for 15min.

Spheroids including medium, in which they were kept in, were transferred to the LEC monolayers and co-incubated at 37°C for 4 h. Pictures of the CCID areas in the LEC monolayers were taken by using an Axiovert (Zeiss, Jena, Germany) fluorescence microscope. Calculation was done with the Zen Little 2012 software

(Zeiss, Jena, Germany). For each condition the CCIDs in the LEC monolayer underneath at least 15 HCT116-spheroids (unless otherwise specified) were measured.

Bay11-7082 dose-response

LECs were seeded in 6-well plates and grown to 80% confluence. Bay11-7082 was added to cells in different concentrations and incubated for 4 h. Cells were harvested and analyzed by Western blotting.

Statistical Analysis

Excel 2013 software and GraphPad Prism 6 (GraphPad, San Diego, CA, USA) were used for Student's t-test statistics. The values were expressed as mean ± SEM. For significance the p- value was set to < 0.05.

Results

12(S)-HETE triggers up-regulation of FAK and SOX18

SOX18 was shown to contribute to cancer cell invasion by up-regulation of mRNA (Fig.2A) as well as protein expression (Fig.2B) when induced by 12(S)-HETE (Fristiohady et al., 2018). FAK was also activated by 12(S)-HETE. Alterations were only observed in phosphorylation at tyrosine 397. The overall FAK protein expression (Fig.2C) did not change. Based on these findings, we investigated the connection between SOX18 and FAK further to find out, whether the two proteins are signal transducers in the same pathway and may cross-talk to each other.

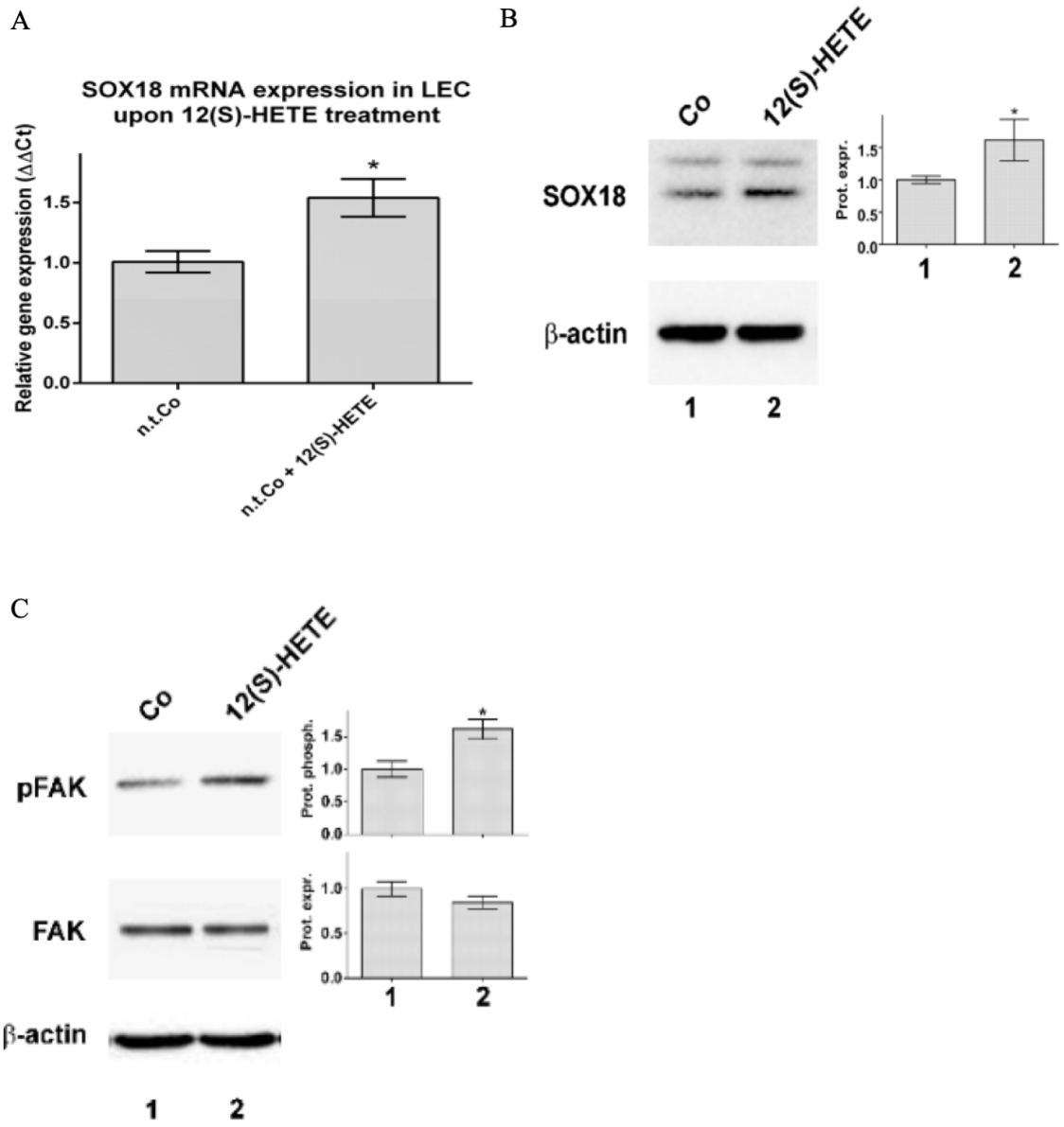

Fig.2: 12(S)-HETE induction up-regulates expression levels of SOX18 and phosphorylation of FAK. Lymph endothelial cells were stimulated with 1µM 12(S)-HETE for 45min. (A) RNA was extracted for reverse transcription and subsequent RT-qPCR to determine SOX18 mRNA expression (B) or analyzed by Western blotting for SOX18 protein expression. (C) FAK levels as well as phosphorylation of FAK (Y397) were analyzed by Western blotting. Densitometry of relative protein concentration was measured with ImageJ. ß-actin and Ponceau S staining served as loading control. All experiments were performed in triplicates. Error bars show standard error of mean. Significance was set to $p<0.05$ and calculated by Student's t-test. Significance is indicated with an asterisk.

SOX18 lies upstream of FAK

FAK is a signaling molecule at the cell membrane upstream of many pathways e.g. MAPK pathway or Akt pathway (Dwyer et al., 2015; Liu et al., 2008; Sonoda et al., 1999). To find out whether FAK is downstream or upstream of SOX18, an RNAi mediated knock-down of FAK was performed which resulted in a decrease of constitutive SOX18 protein (Fig.3A) as well as mRNA expression (Fig.3B). Knock-down efficiency of FAK is shown in Supplement Figure S.1A.

In addition, SOX18 could not be activated by 12(S)-HETE (Fig.3C) when FAK levels were inhibited, supporting the theory that FAK is needed for 12(S)-HETE induced signal transduction to SOX18 and thus, lies upstream of SOX18.

Also, constitutive expression of PROX1, the bona fide target of SOX18, was downregulated when FAK was knocked down. Interestingly however, 12(S)-HETE triggered up-regulation of PROX1 was not affected at all (Fig.3D). A very different outcome could be observed, when SOX18 was inhibited. This time, 12(S)-HETE-triggered induction of PROX1 was abolished upon inhibition of SOX18 (Fig. 3E), suggesting that SOX18 was activated by 12(S)-HETE at first instance and therefore lied upstream of FAK.

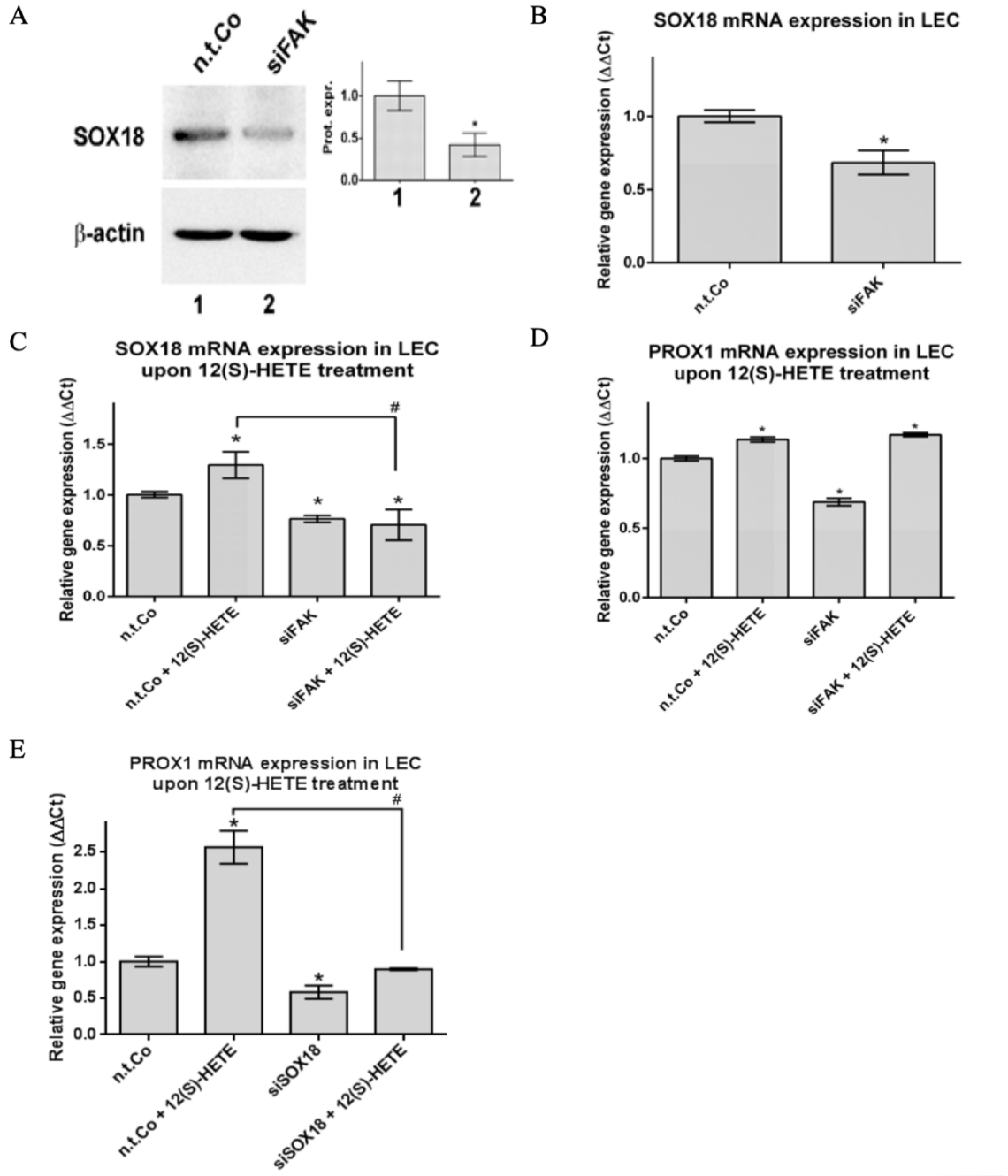

Fig.3: FAK positively regulates SOX18 and its downstream target PROX1. Lymph endothelial cells were transiently transfected with siRNA targeting FAK (siFAK) or non-target siRNA (n.t.Co). (A) SOX18 protein concentration was determined by Western blotting or (B) RNA was extracted and reverse transcribed to analyze SOX18 mRNA levels via RT-qPCR. Additionally, transfected LECs were stimulated with 1µM 12(S)-HETE for 45min to determine gene expression of (C) SOX18 and (D) PROX1 analyzed by RT-qPCR. (E) LECs were transiently transfected with siRNA targeting SOX18 (siSOX18) or non-target siRNA (n.t.Co). mRNA levels of PROX1 were obtained by RT-qPCR. Densitometry of relative protein concentration was analyzed with ImageJ. ß-actin and Ponceau S staining served as loading control. Experiments were performed in triplicates. Error bars show standard error of mean. Significance was evaluated using the Student's t-test with $p<0.05$. Significance is indicated with an asterisk or hash tag.

FAK and SOX18 form a feed-back loop

To test this hypothesis SOX18 was knocked down, which increased phosphorylation of FAK, but not the protein expression (4A). The knock-down efficiency of SOX18 on the protein and mRNA level was additionally evaluated. (S.1B & S.1C). Stimulation with 12(S)-HETE failed to induce phosphorylation of FAK when SOX18 was inhibited (Fig.4B). This confirmed that SOX18 was upstream of FAK.

In conclusion, a feed-back loop between SOX18 and FAK was found to keep constitutive expression of FAK as well as SOX18 at a moderate level (Fig.4C). Upon stimulation with 12(S)-HETE, SOX18 was required for phosphorylation of FAK. At the same time FAK secured proper expression of SOX18 and augmented up-regulation of SOX18 upon 12(S)-HETE induction.

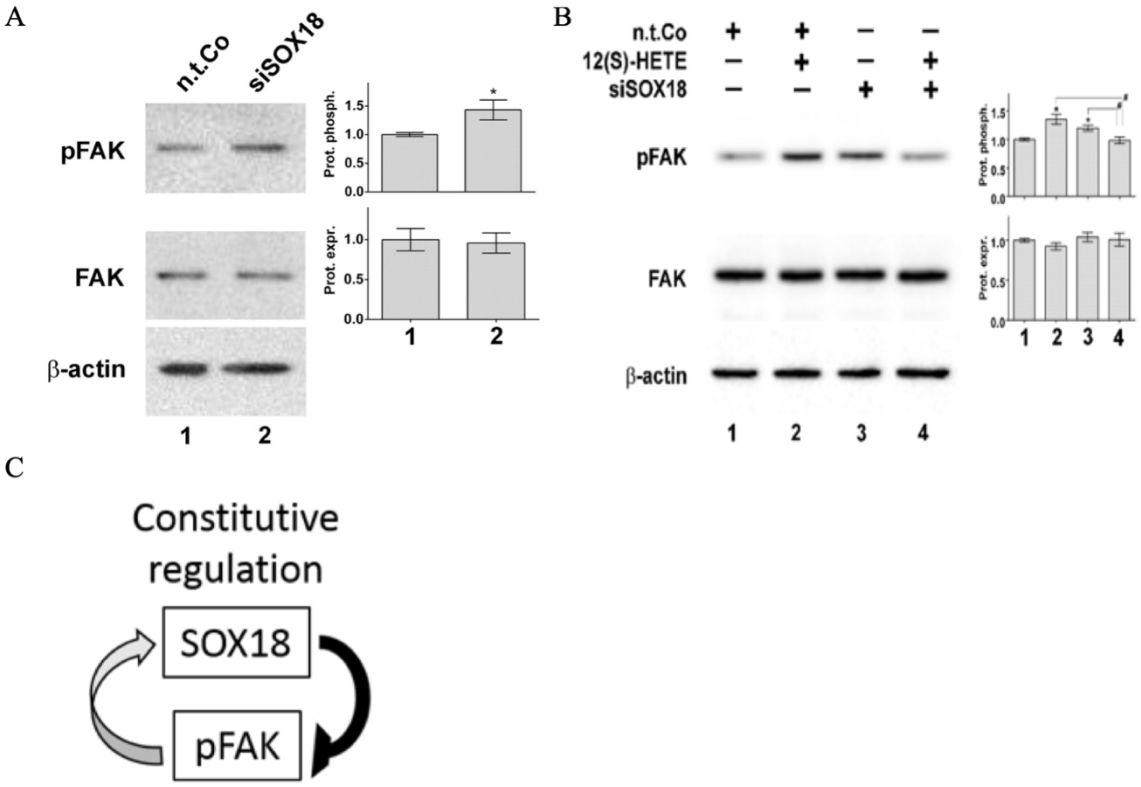

Fig.4: SOX18 lies upstream of FAK. Lymph endothelial cells were transiently transfected with siRNA targeting SOX18 (siSOX18) or non-target siRNA (n.t.Co). (A) FAK protein expression and level of phosphorylated FAK (Y397) under constitutive cell culture conditions were analyzed by Western blotting. (B) Additionally, transfected LECs were stimulated with 1µM 12(S)-HETE for 45min. Total protein expression of FAK and phosphorylation of FAK (Y397) were obtained by Western blotting. Relative protein concentration was measured with ImageJ. ß-actin and Ponceau S staining served as loading control. Error bars show standard error of mean. Significance was calculated with the Student's t-test with $p<0.05$. Significance is indicated as asterisk or hashtag. Experiments were performed in triplicates. (C) Scheme: Feed-back loop between SOX18 and FAK in which SOX18 regulates activation of FAK negatively. In turn, FAK regulates SOX18 expression positively under constitutive cell culture conditions.

RELA and SOX18 are interconnected

RELA is the most abundant protein in the heterodimeric NF-κB complex and was induced by 12(S)-HETE (Fig.5A). In the next step, we were interested, whether NF-κB signaling was linked to the SOX18-FAK loop. It was recently discovered that SOX18 and NF-κB signaling are interconnected (Basílio et al., 2013; Fristiohady et al., 2018). The downregulation of SOX18 upon RELA knock-down was demonstrated in LECs (Fig.5B). As expected PROX1 showed similarly reduced levels in response to the RELA inhibition (Fig.5C). Hence, RELA influenced SOX18 and PROX1 expression. Knock-down efficiency of RELA on the protein level is shown in Figure 5A. Additionally, knock-down efficiency of RELA on the mRNA level was evaluated (S.1D).

Conversely, to check the effect of SOX18 on RELA, SOX18 was inhibited by using siRNA resulting in downregulation of RELA (Fig.5D). When stimulating SOX18-siRNA transfected cells with 12(S)-HETE, 12(S)-HETE induction of RELA was abrogated (Fig.5D). Additionally, the expression of ICAM-1, a proven bona fide target of NF-κB signaling, was analyzed (Viola et al., 2013). The 12(S)-HETE-triggered induction of ICAM-1 was prevented upon SOX18 knock-down, whereas constitutive expression did not change. In conclusion, SOX18 and RELA mutually controlled each other in a positive feed-back loop under constitutive culture conditions. (Fig.5F) Furthermore, SOX18 was required for 12(S)-HETE-mediated up-regulation of RELA, which positively fed back to SOX18.

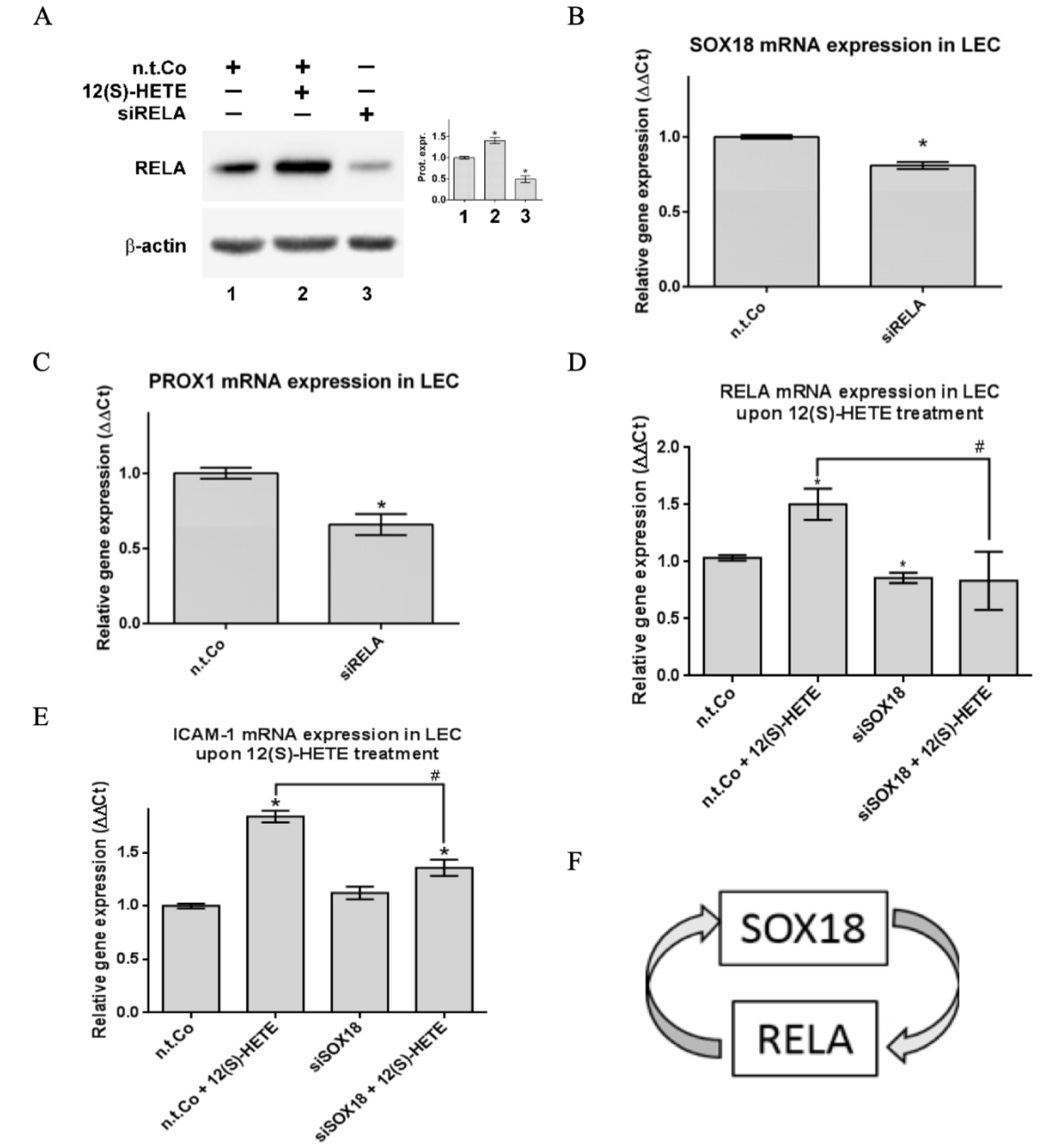

Fig.5: RELA and SOX18 form a positive feedback loop. Lymph endothelial cells (LECs) were transiently transfected with siRNA targeting RELA (siRELA) or non-target siRNA (n.t.Co) and (A) stimulated with 1µM 12(S)-HETE for 45min followed by cell lysis for Western blot analysis. ß-actin and Ponceau S staining served as loading control. Densitometry of relative protein concentration was measured with ImageJ. For mRNA expression of (B) SOX18 and (C) PROX1, RNA was extracted, reverse transcribed and analyzed by RT-qPCR. LECs were transiently transfected with siRNA targeting SOX18 (siSOX18) or non-target RNA (n.t.Co) and stimulated with 1 µM 12(S)-HETE for 45 min. The gene expression of (D) RELA and (E) ICAM-1 was analyzed by RT-qPCR. Error bars represent the standard error of mean. Significance was measured using the Student´s t-test with $p<0.05$. Significance is indicated with an asterisk or hashtag. Experiments were performed in triplicates. (F) Scheme: RELA and SOX18 form a positive feed-back loop.

RELA regulates FAK activation

FAK and NF-κB are both reported to regulate and activate each other (Wang et al., 2014; Zhang et al., 2006). In LECs, FAK did not have any influence on RELA expression levels under constitutive conditions (Fig.6A). However, upon treatment with 12(S)-HETE, the up-regulation of RELA was prevented when FAK was knocked down (Fig.6B). Also, ICAM-1 showed no up-regulation upon 12(S)-HETE induction when FAK was inhibited (Fig.6C). Thus, constitutive expression of RELA and further the NF-κB signaling pathway was independent of FAK (Fig.6D). Nonetheless, FAK was pivotal for 12(S)-HETE triggered induction of RELA.

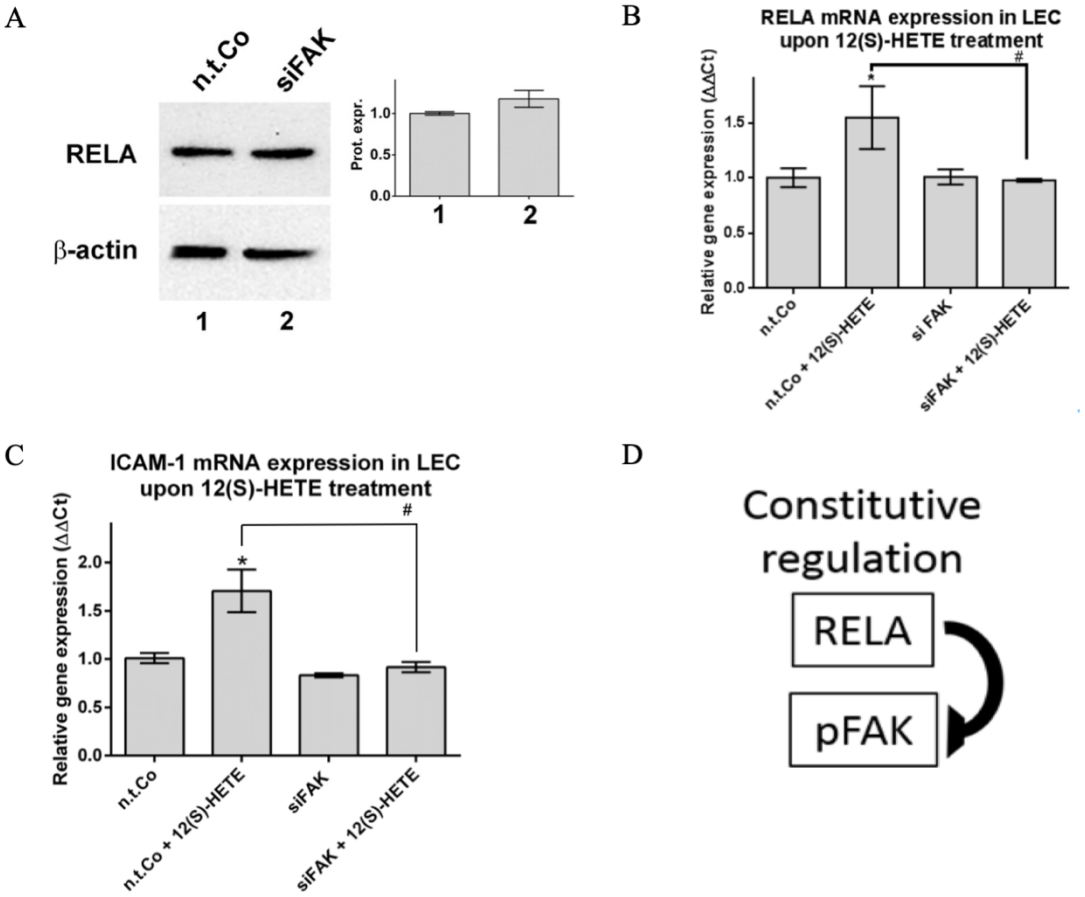

Fig.6: FAK has no effect on NFkB signaling. Lymph endothelial cells (LECs) were transiently transfected with either non-target siRNA (n.t.Co) or siRNA targeting FAK (siFAK). (A) Protein concentration of RELA was obtained by Western blotting. Densitometry of relative protein concentration was measured with ImageJ. ß-actin and Ponceau S staining served as loading control. (B) For RELA mRNA expression and (C) ICAM-1 mRNA expression transfection of LECs with either siRNA targeting FAK (siFAK) or non-target siRNA (n.t.Co) was followed by stimulation with 1µM 12(S)-HETE and measured by RT-qPCR. Error bars represent standard error of mean. Significance was evaluated using the Student´s t-test with $p<0.05$ and indicated with an asterisk or hashtag. All experiments were performed in triplicates. (D) Scheme: RELA controls constitutive expression of FAK negatively, whereby FAK has no effect on the constitutive expression of RELA.

Surprisingly, when knocking-down RELA, phosphorylation of FAK was induced, whereas total FAK expression did not undergo any changes. Hence, RELA seemed to be a key player in downregulating FAK activity since FAK phosphorylation increased when RELA was suppressed both under constitutive and 12(S)-HETE-triggered conditions (Fig.7A). This could also be confirmed by using Bay11-7082, an irreversible inhibitor of IKK-α phosphorylation in NF-κB signaling. Testing different concentrations ranging from 0-15 μM, FAK appeared to be hyperphosphorylated and FAK protein levels were upregulated in a concentration dependent manner (Fig.7B).

All three molecules, SOX18, RELA and FAK, were induced by 12(S)-HETE and proved to be in the same signal transduction network. SOX18 and RELA regulated phosphorylation of FAK negatively. Upon stimulation with 12(S)-HETE SOX18 was activated first and promoted the activation of RELA and FAK. In return, RELA and FAK enhanced SOX18 up-regulation by positively feeding back to it. Under constitutive expression conditions RELA was independent of FAK, but formed a positive feed-back loop with SOX18 (Fig.7C).

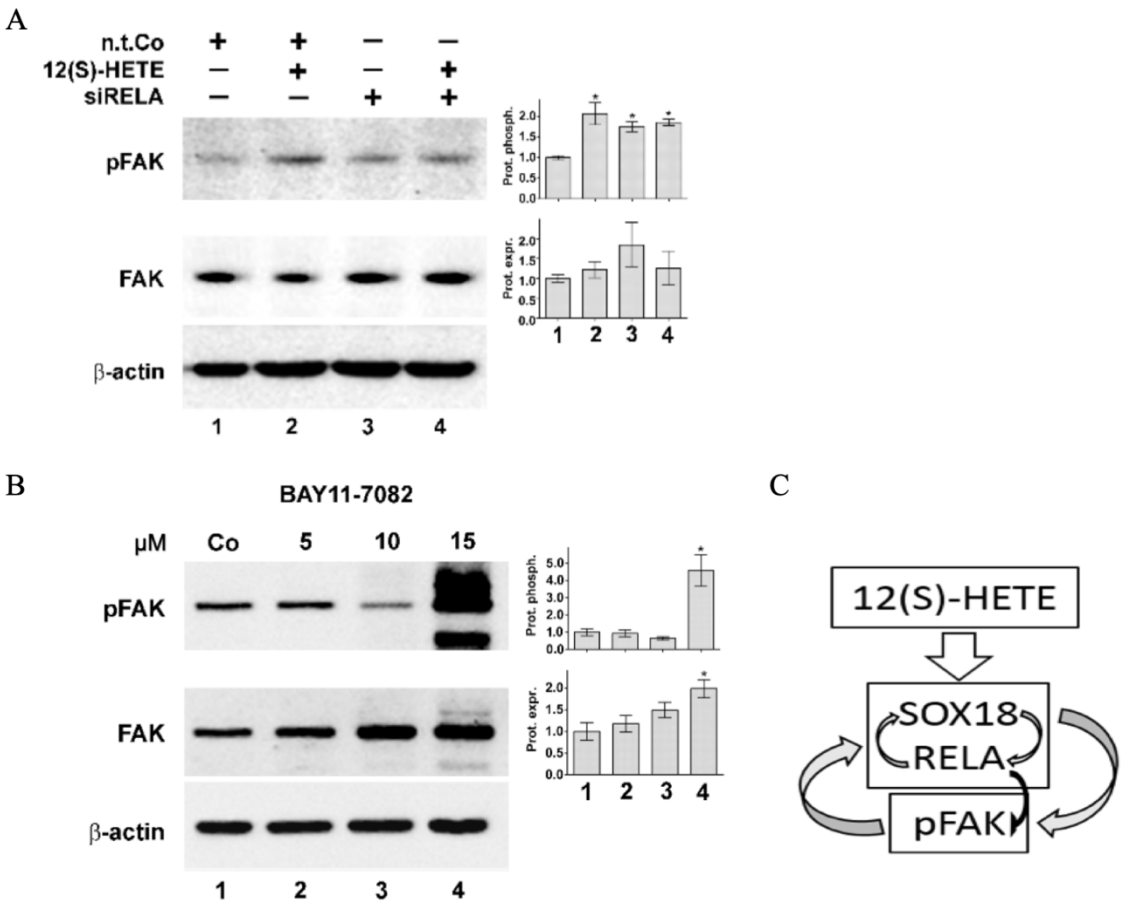

Fig.7: Phosphorylation levels of FAK were increased when RELA was inhibited. (A) LECs were transiently transfected with siRNA targeting RELA (siRELA) or non-target siRNA (n.t.Co) followed by stimulation with 1μM 12(S)-HETE for 45min. Results were obtained by Western blotting. (B) LECs were pre-treated with solvent (DMSO, Co) or Bay11-7082 with a concentration ranging from 5-15μM, incubated for 4h at 37°C and analyzed by Western blotting. Experiments were performed in triplicates. Densitometry of relative protein concentration was measured with ImageJ. Error bars show standard error of mean. ß-actin and Ponceau S staining served as loading control. (D) Scheme: RELA is involved in the FAK-SOX18 feed-back circuit by positively feeding back to SOX18 and regulating FAK activation negatively.

Inhibition of lymph endothelial barrier breaching by molecular silencing

HCT116 colon cancer cells were found to express ALOX12 and CYP1A1 as potential 12(S)-HETE producers. (Honn et al., 1994; Nguyen et al., 2016a) Kerjaschki and co-workers provided evidence that ALOX12 do not produce sufficient levels of 12(S)-HETE in this cell line (Kerjaschki et al., 2011). Analysis by ELISA resulted in high levels of 12(S)-HETE when stimulated with arachidonic acid (Fig.8A). Additionally, cells were treated with three different CYP1A1 inhibitors guanfacine, proadifen, and vinpocetine (Holzner et al., 2018; Teichmann et al., 2014), which all showed a significant inhibition of metabolized 12(S)-HETE. Thus, providing evidence that CYP1A1 was the main producer of 12(S)-HETE in HCT116 colon cancer cells.

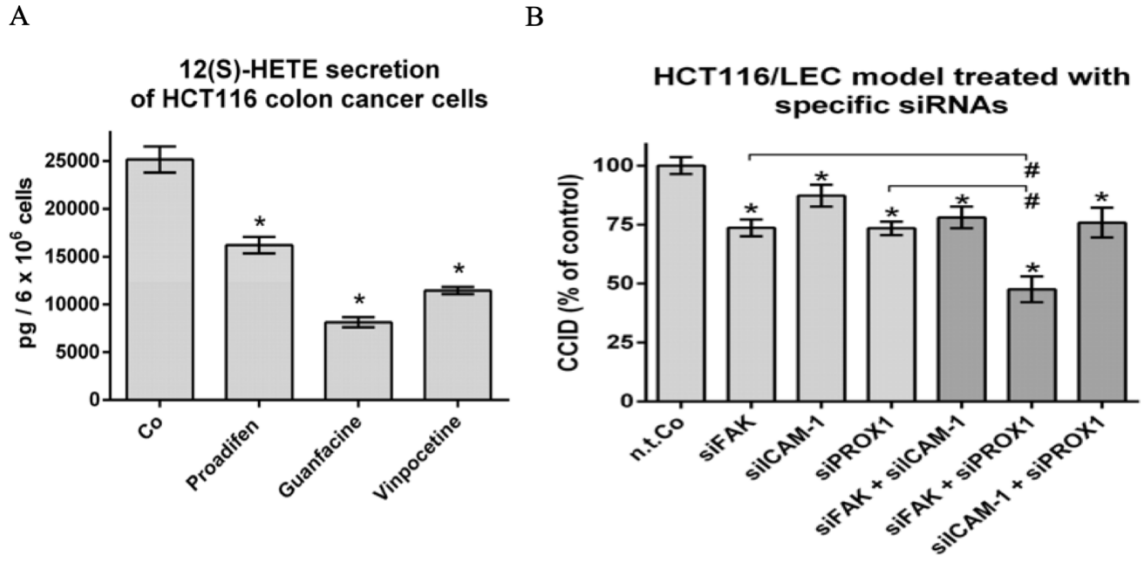

Fig.8: RELA, SOX18 and FAK are key players in cell barrier breaching. (A) HCT116 colon cancer cells were stimulated with 10µM arachidonic acid and simultaneously with 40µM proadifen, guanfacine or vinpocetine. DMSO served as control. 12(S)-HETE was extracted from supernatant and concentration determined with an immune assay kit. Values are normalized to cell number and given in picograms. (B) Lymph endothelial cells (LECs) were transiently transfected with either siRNA targeting FAK (siFAK), ICAM-1 (siICAM-1), PROX1 (siPROX1) or non-target siRNA (n.t.Co) and further in combination. After 24h HCT116 colon cancer spheroids were placed on a confluent lymph endothelial monolayer and co-incubated for 4h at 37°C. Analysis was done by using an Axiovert microscope and Zen Little 2012 software. Experiments were conducted in triplicates with at least 5 replicates per sample. Significance was evaluated using the Student´s t-test with $p<0.05$ and indicated with an asterisk or hashtag. Error bars represent the standard mean of error.

To investigate, whether inhibitors of the discovered feed-back loop may impair cancer intravasation, PROX1 as target of SOX18 signaling, ICAM-1 as target of NF-κB signaling, and FAK were co-transfected into LECs and further used in the CCID assay.

Inhibition of PROX1 and FAK reduced CCID formation around 25%, whereas inhibition of ICAM-1 attenuated CCID formation by only ~12%. PROX1 & FAK showed a combined effect in reducing CCID formation by over 50%. However, PROX1 & ICAM-1 and FAK & ICAM-1 reduced CCIDs by only ~20%, which was similar to the inhibitory effects of FAK and PROX1 alone (Fig.8B). The obtained results confirmed our findings that FAK was interconnected with SOX18 and played a pivotal role in cancer cell intravasation. Furthermore, RELA may have served as a common link between FAK and SOX18.

Inhibition of lymph endothelial barrier breaching by pharmacological drugs

Currently there are no treatments with siRNA available. Therefore, we tested the activity of curcumin and parthenolide (Fig.9A) as naturally occurring NF-κB inhibitors and defactinib as clinical trial phase II inhibitor of FAK (Fig.9B) with the CCID assays. LEC monolayers and HCT116 spheroids were pre-incubated with different concentrations of the compounds and subsequently co-cultured for 4 h by placing spheroids on the monolayers. CCID formation was significantly reduced for all three compounds in a concentration dependent manner.

In the next step, HCT116 spheroids were pre-incubated with defactinib and Bay11-7082 separately and in combination followed by a 4 h incubation with spheroids placed on the LEC monolayer. Both inhibitors showed attenuation of CCID formation around 25% on their own. In combination, they exhibited an even stronger effect in inhibiting CCID formation and reduced CCIDs by ~40% (Fig.9C). Accordingly, FAK and NF-κB may have a joined signaling route.

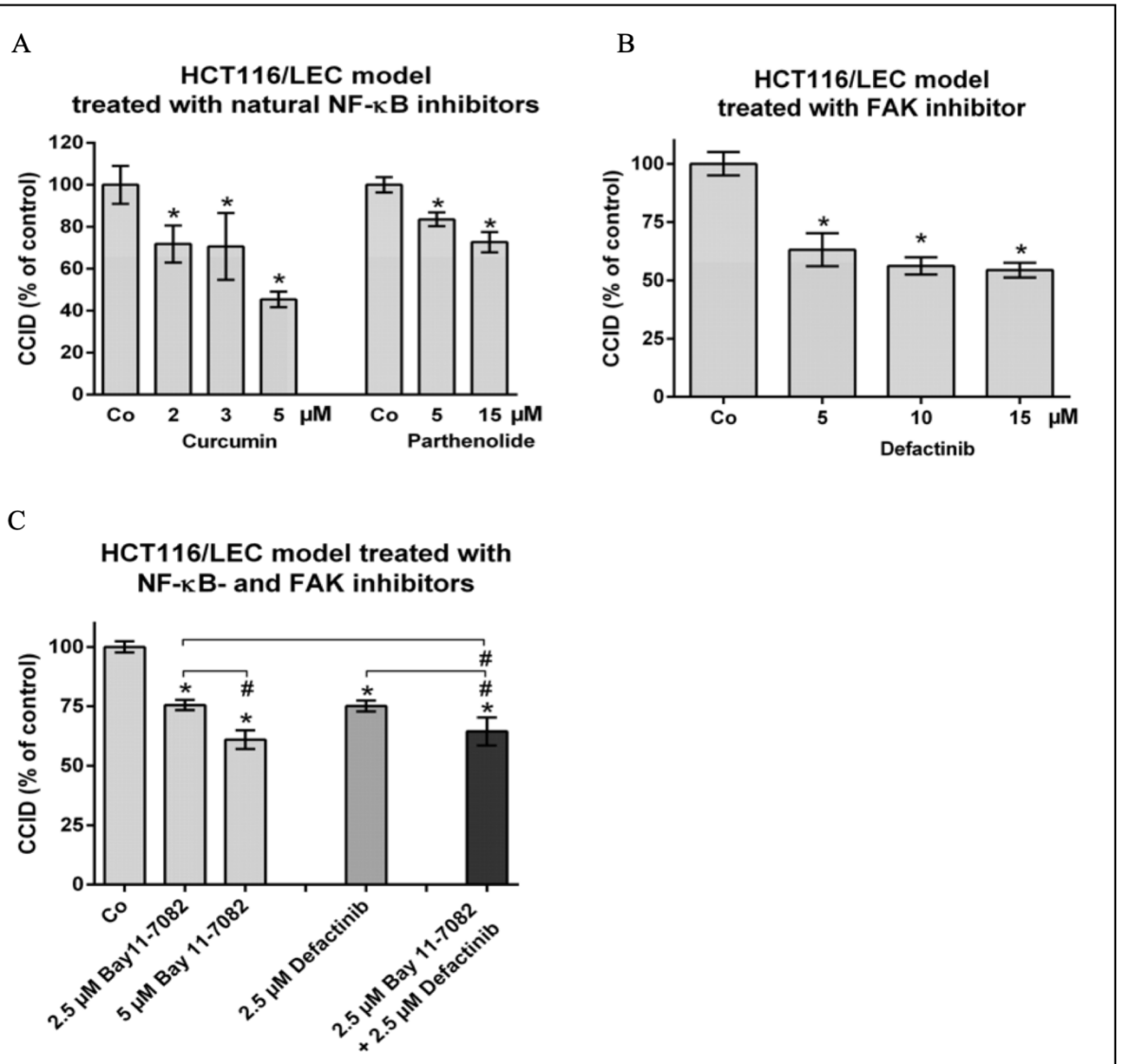

Fig.9: Curcumin, parthenolide, Bay11-7082 and defactinib are potent inhibitors to reduce CCID formation. Lymph endothelial cells and HCT116 colon cancer spheroids were pre-treated with solvent (DMSO, Co) or different concentrations of (A) curcumin & parthenolide as natural NFkB-inhibitors or (B) defactinib as clinical trial phase II FAK inhibitor for 20min. Spheroids were placed on a confluent lymph endothelial monolayer and co-incubated for 4h. (C) Lymph endothelial cells were pre-treated with either solvent (DMSO, Co), Bay 11-7082, defactinib or in combination for 15min and co-incubated with spheroids for 4h. Analysis was done by using an Axiovert microscope and Zen Little 2012 software. Experiments were conducted in triplicates with at least 5 replicates per sample. Significance was evaluated using the Student´s t-test with $p < 0.05$ and additionally by One-way ANOVA. Significance is indicated with an asterisk or hashtag. Error bars represent the standard mean of error.

Discussion

Cell barrier breaching is a crucial step for tumor intravasation and extravasation and therefore a hallmark in early tumorigenesis (Kerjaschki et al., 2011). Recently it was discovered that SOX18 and PROX1 are key players in retraction of lymph endothelial cells induced by 12(S)-HETE (Basílio et al., 2013; Fristiohady et al., 2018). 12(S)-HETE is a metabolite released by cancer cells enabling intravasation and further seems to be crucial in endothelial to mesenchymal transition (endo-MT). Endo-MT is a process of changing cell specific properties by which the cell barriers are loosened, giving cancer cells access to lymphatic or blood vessels. By inducing the expression of cell adhesion proteins, ICAM-1 was identified as an effector molecule upon 12(S)-HETE stimulation on the surface of LECs through which the tumor can dock onto the lymph endothelium (Viola et al., 2013). LECs undergo morphological as well as expressional changes. In course, endothelial markers e.g. VE-cadherin are repressed and expression of mesenchymal markers such as S100A4 or motility proteins such as MLC2 are upregulated. Showcasing the ability of 12(S)-HETE supports the hypothesis of being an endo- MT trigger (Nguyen et al., 2016; Vonach et al., 2011).

Moreover, 12(S)-HETE seems to be a potential factor to prepare tissues at distant sites since molecules deregulated in endo-MT also promote metastatic niche formation. One of the most important markers identified was VEGF in bone marrow cells which is not only controlled by 12(S)-HETE, but was also found to be influenced by FAK especially in angiogenesis (Kaplan et al., 2005; Tavora et al., 2010). Additionally, FAK was suggested to be responsible for up-regulation of mesenchymal markers and cleavage of E-cadherin from the cell surface in TGFß-mediated EMT of hepatocytes (Cicchini et al., 2008).

The involvement of SOX18 and FAK in several steps of tumor progression such as migration, intra- and extravasation, and angiogenesis makes them likely factors to participate in pre-metastatic niche forming as well (Houg and Bijlsma, 2018).

Cancer cells do not only control gene expression of the colonized organism to their advantage, but also release different stimuli to accelerate cancerous processes. MMP1 signaling was found to be activated by NF-κB in MDA-MB231 breast cancer cells which binds to the PAR1 receptor expressed on LECs. Binding resulted in phosphorylation of FAK leading to LEC retraction (Nguyen et al., 2015). In preliminary experiments we also showed that MMP1 could be another potential trigger for SOX18 activation. It is still unclear what kind of downstream processes are elicited and how they will be influenced by this stimulus. By now, the only reported interaction between SOX18 and matrix metallo proteases is regulation of MMP7 by SOX18 (Hoeth et al., 2012).

Key players of developmental pathways are often deregulated driving carcinogenesis. SOX18, RELA and FAK are such key players which all were linked to cancerous processes and also promoted barrier disintegration (Sulzmaier et al., 2014; Thu et al., 2014; Xia et al., 2018). In this thesis, a novel feed-back circuit is described that unites these three factors in a common signaling network. It is shown for the first time that the cancer-secreted metabolite 12(S)-HETE activated FAK in LECs. SOX18 was the first of these signal transducers reached upon 12(S)-HETE treatment, placing it upstream of FAK. This finding was confirmed by the independence of PROX1 up-regulation upon transfection with siFAK. Furthermore, RELA expression was increased upon 12(S)-HETE-induced up-regulation of SOX18 and positively fed back to SOX18 maintaining a steady signal nourishing FAK phosphorylation at tyrosine 397. FAK closed the circle by positively feeding back to SOX18 as well. Constitutive expression of FAK was negatively regulated by SOX18 and RELA controlling expression levels. However, FAK had no effect on constitutive RELA expression, but formed a feed-back circuit with SOX18. Our findings imply that the SOX18 feed-back loop served as on/off switch for rapid activation or shut down of FAK expression upon stimulation with 12(S)-HETE. Since SOX18 and RELA are transcription factors, the phosphorylation of FAK must have occurred by a yet unidentified tyrosine kinase.

To demonstrate the effect of the findings derived from the 2D experiments in LECs, we used the CCID assay as a 3D model of intravasation to test the combined effect of the respective siRNAs and furthermore of pharmacological relevant compounds that can reduce retraction and subsequent migration of LECs.
In the CCID assay, effectors of SOX18 and RELA in addition with FAK were tested. A combination of PROX1 & ICAM-1 inhibition (whereas PROX1 is the downstream target of SOX18 and ICAM-1 the downstream target of RELA) and FAK & ICAM-1 inhibition did not show any additional inhibitory effect compared to the inhibitory efficiency of FAK, PROX1 or ICAM-1 alone. Hence, FAK & NF-κB or SOX18 & NF-κB seemed to reside in the same signaling pathway. Interestingly, PROX1 & FAK knock-down had an additive inhibitory effect on LEC retraction. This suggested that SOX18/PROX1 and FAK, nevertheless cross-talking to each other, played profoundly different roles regarding the retraction of LECs. siRNAs block translation of a gene, therefore preventing protein expression first and foremost. This makes them a highly specific tool, but are not yet available for treatment.

Therefore, we tested curcumin and parthenolide, which are naturally occurring NF-κB inhibitors, reducing CCID formation up to 50% at low concentrations. Also, Bay11-7082, a well-known inhibitor of NF-κB signaling, and defactinib, a FAK inhibitor, significantly reduced LEC retraction. In combination defactinib and Bay11-7082 reduced CIDD formation almost twice as much as each inhibitor on its own, assuming NF-κB signaling and FAK signaling have a combined effect. However, the additive effect may rather occur due to additional effects of the inhibitor on other molecules since kinase inhibitors are often directed towards the ATP binding site and therefore lacking specificity.

Nonetheless, defactinib holds great promise as specific FAK inhibitor. The here used inhibitor was created by combining two FAK inhibitors in one (PF-04554878 &VS-6063), disrupting kinase function. Defactinib was listed in 15 studies to test its effect on malignancies. Five trials have been completed successfully, the most advanced being a phase II trial (NCT01951690). Three of the studies were terminated for reasons unknown. Results have not been published yet, but the outlook for defactinib seems to be optimistic since 7 other studies are recruiting.

Most inhibitors target protein/DNA binding or intervene with post-translational modifications. (Fontaine et al., 2017). Transcription factors often activate gene expression by protein-protein interaction instead of protein/DNA binding. They are considered promiscuous due to activation of many different targets which holds difficulties for safety of inhibitors. Through the lack of binding pockets or defined three dimensional structures of transcription factors, development of inhibitors proves to be difficult. For SOX18 different attempts have been made to find suitable SOX18 inhibitors by targeting protein-protein interaction with extracts from the brown algae Caulocystis cephalornithos Sm1, Sm2 and Sm4. They were reported to disrupt the interaction between SOX18 and binding partners and additionally able to interfere with the DNA binding domain (Francois et al., 2018; Overman et al., 2017). Also blocking special motifs within the DIM domain to disrupt homodimerization were considered (Moustaqil et al., 2018). All in all, some options for SOX18 inhibitors do exist, but are still in the initial phase and need to be further investigated.

In conclusion, SOX18 together with FAK seem to have a drastic effect on cancer progression at an early stage. Defactinib as a clinical trial phase II inhibitor is exhaustingly tested because FAK inhibition in combination with other drugs or chemotherapy showed good results (Rožanc et al., 2018; Tavora et al., 2014). However, regarding the NF-κB pathway and FAK, targeting must be treated with caution due to their position as central signal transducers controlling many different targets. Conversely, SOX18 and its target PROX1 would be preferred candidates since they are uniquely involved in endothelial differentiation and lymph endothelial maintenance. They may provide the specificity urged by blocking cancer dissemination through lymphatics, as this may reduce adverse effects and toxicity.

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Appendix

List of figures

Abstract Deutsch

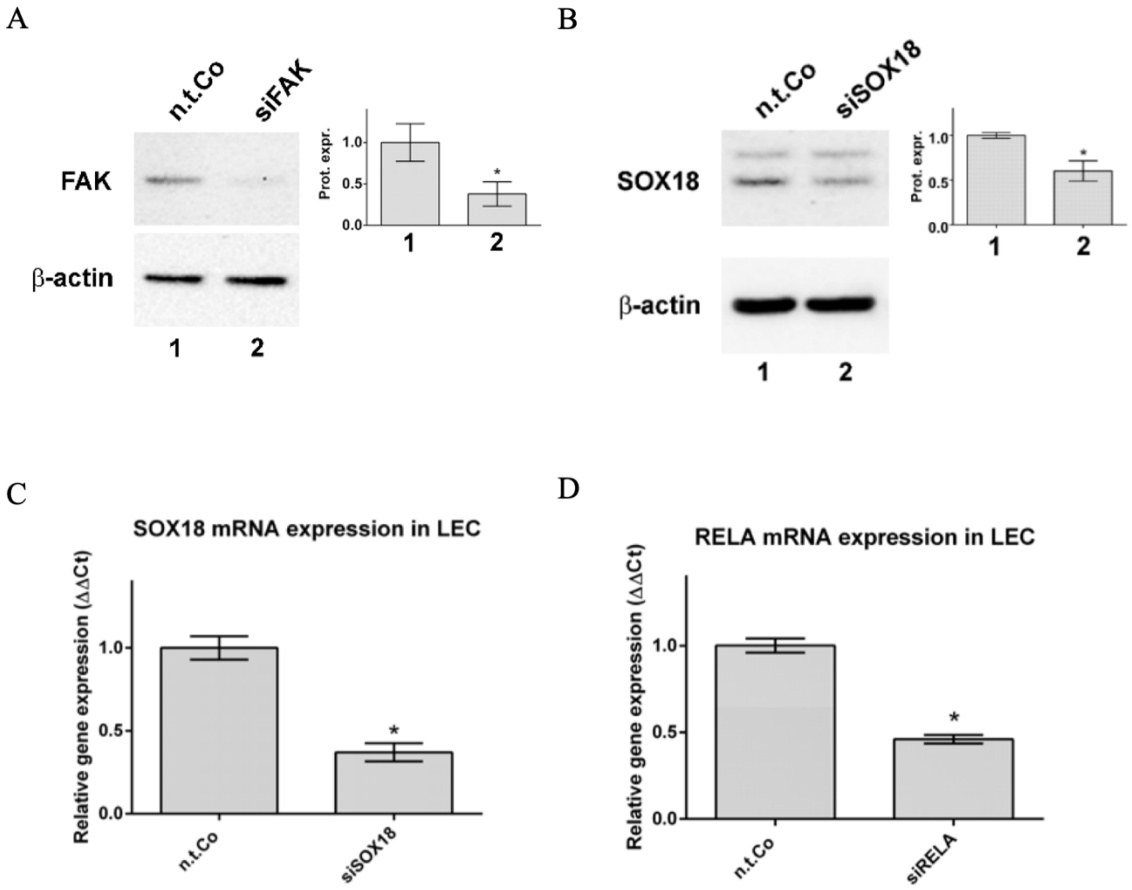
12(S)-HETE ist ein Molekül, das von Krebszellen freigesetzt wird, um das Einwandern durch das Endothel von Blut- und Lymphgefäßen zu ermöglichen. Der Transkriptionsfaktor SOX18 spielt eine wichtige Rolle in der Differenzierung des Endothels und wurde kürzlich in Zusammenhang mit Retraktion des Lymphendothels gebracht. FAK ist eine Kinase, die an den Fokalkontakten der Zelle sitzt, und in vielen Krebsarten dereguliert ist. Weiters ist bekannt, dass FAK in Zellmigration involviert ist. Deswegen stellte sich die Frage, ob FAK und SOX18 im gleichen Signalweg arbeiten. Es wurde überprüft, ob Induktion mit 12(S)-HETE die Aktivität von FAK beeinträchtigt. Zusätzlich wurde getestet, welchen Einfluss NF-κB auf die SOX18-FAK Interaktion nimmt. Lymphendothelzellen wurden mit siRNA transfektiert, um Expressionslevel sowohl auf Proteinebene als auch auf RNA Ebene ermitteln zu können. So wurde ein Signalnetzwerk gefunden, das als Ein- und Aus-Schalter für FAK Aktivität dient. Da siRNAs im medizinischen Bereich als Therapiemethode noch nicht eingesetzt werden, wurden zwei in der Natur vorkommende NF-κB-Inhibitoren, Curcumin und Parthenolid, sowie ein FAK-Inhibitor, der bereits klinisch getestet wird, namens Defactinib, auf deren anti-intravasive Eigenschaften getestet. Hierfür wurde der „Circular chemorepellent induced defects“ (kurz: CCID) Assay eingesetzt. Dieser schafft eine 3D Umgebung, die den Intravasationsprozess im Körper nachstellt. Dabei werden aus Krebszellen Spheroide gezüchtet und auf einen einfachen Lymphendothelzelllayer gesetzt. Nach 4 Stunden konnte für alle Inhibitoren und auch Kombinationen ein Rückgang an im Lymphendothel entstehenden CCIDs festgestellt werden. CCIDs entstehen durch Substanzen, die Krebszellen ausschütten, und damit die Wanderung von Lymphendothelzellen auslösen. Zusammengefasst bedeutet das, dass RELA, FAK und SOX18 wichtige Komponenten für die Retraktion von Lymphendothelzellen darstellen und somit Tumorintravasation erleichtern.

Abbreviations

12(S)-HETE	12S-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid, "S" stereoisomer
12-HETER	12(S)-HETE receptor (syn. GPR31)
BLT2	Leukotriene B4 receptor 2 (syn. BLT2 receptor, BLT2R, low affinity LTB4 receptor)
CCID	Circular chemo repellent induced defect
CYP1A1	Cytochrome P450 1A1
DMSO	Dimethyl sulfoxide
EC	Endothelial cell
Endo-MT	Endothelial to mesenchymal transition
FAK	Focal adhesion kinase
ICAM-1	Intercellular adhesion molecule 1
IKK-α	IκB kinase-α (also IKK-1)
LEC	Lymph endothelial cell
MLC2	Myosin light chain 2
mRNA	messenger ribonucleic acid
MYLK	Myosin light chain kinase
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAR1	Protease activated receptor 1
PROX1	Prospero homeobox protein 1

RELA	V-Rel avian reticuloendotheliosis viral oncogene homolog A
RT-qPCR	Real Time- quantitative Polymerase Chain Reaction
siRNA	small interfering ribonucleic acid
SOX18	SRY-related HMG-box 18

Supplement figure 1



S.1: Knock down efficiency of FAK, SOX18 and RELA. (A) LECs were transfected with 1,9µg of siRNA targeting FAK (siFAK) or non-target siRNA (n.t.Co) using Hiperfect. Results were obtained with Western blotting. (B) LECs were transfected with 1,9µg of siRNA targeting SOX18 (siSOX18) or non-target siRNA (n.t.Co) using Hiperfect. Results were obtained with Western blotting or (C) RT-qPCR. (D) LECs were transfected with 1,9µg of siRNA targeting RELA (siRELA) or non-target siRNA (n.t.Co) using Hiperfect. To evaluate the mRNA level results were obtained with RT-qPCR. All experiments were executed three times. For Western blotting ß-actin and Ponceau S staining served as loading control. Densitometry of relative protein concentration was analyzed with ImageJ. Error bars show standard error of mean. Significance was calculated with the Student's t-test with $p<0.05$. Significance is indicated with an asterisk.