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Endothelial cell-fatty acid binding protein 4 promotes angiogenesis: role of stem cell factor/c-kit pathway

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Abstract

Fatty acid binding protein 4 (FABP4) plays an important role in regulation of glucose and lipid homeostasis as well as inflammation through its actions in adipocytes and macrophages. FABP4 is also expressed in a subset of endothelial cells, but its role in this cell type is not known. We found that FABP4-deficient human umbilical vein endothelial cells (HUVECs) demonstrate a markedly increased susceptibility to apoptosis as well as decreased migration and capillary network formation. Aortic rings from FABP4^{-/-} mice demonstrated decreased angiogenic sprouting, which was recovered by reconstitution of FABP4. FABP4 was strongly regulated by mTORC1 and inhibited by Rapamycin. FABP4 modulated activation of several important signaling pathways in HUVECs, including downregulation of P38, eNOS, and stem cell factor (SCF)/c-kit signaling. Of these, the SCF/c-kit pathway was found to have a major role in attenuated angiogenic activity of FABP4-deficient ECs as provision of exogenous SCF resulted in a significant recovery in cell proliferation, survival, morphogenesis, and aortic ring sprouting. These data unravel a novel pro-angiogenic role for endothelial cell-FABP4 and suggest that it could be exploited as a potential target for diseases associated with pathological angiogenesis.

Keywords

Angiogenesis; Endothelial cells; FABP4; Rapamycin; c-Kit; Stem cell factor

Introduction

Angiogenesis, the process of forming new blood vessels from existing blood vessels through sprouting and budding of new capillaries, is an essential process in many physiological as well as pathological conditions, including development, reproduction, wound-healing and tumor growth [1–3]. Endothelial cells (ECs) play a crucial role in all stages of angiogenesis, which involves a variety of coordinated events including migration and proliferation of ECs, lumen formation, and anastomosis of sprouts. VEGF signaling pathway, which is recognized as one of the key regulators of angiogenesis, has been the primary target of anti-angiogenic therapies [4]. However, the clinical use of VEGF blockers, particularly in patients with cancer, has been faced with major challenges, including modest improvement in overall survival in several cancer types as well as toxicity [4, 5]. Hence, there is an ongoing need for identification of novel anti-angiogenic targets for treatment of pathological angiogenesis in cancer and other angiogenesis-dependent diseases.

Fatty acid binding protein 4 (FABP4, adipocyte-FABP, aP2) is a member of the family of intracellular FABPs [6]. The FABP family consists of 9 highly conserved, small molecular weight (~15 kDa) cytosolic proteins that are abundantly expressed in a tissue-specific manner with some overlaps [7]. FABPs are capable of binding a variety of hydrophobic ligands, such as long-chain fatty acids, eicosanoids, leukotrienes and prostaglandins [8, 9]. FABP4 plays an important role in regulation of glucose and lipid homeostasis as well as inflammation through its actions in adipocytes and macrophages. The biological relevance of FABP4 is underscored by the findings that FABP4 knock-out (FABP4^{-/-}) mice exhibit marked protection against insulin resistance, atherosclerosis, fatty liver disease, and asthma [8, 10–14]. Consistent with these studies, a small-molecule inhibitor of FABP4 was found to be an effective therapeutic agent in treatment of atherosclerosis and diabetes in mouse models [15]. FABP4-deficient macrophages exhibit decreased inflammatory activity, increased activation of PPAR γ and decreased NF κ B signaling [16]. In accordance with these in vitro observations, FABP4^{-/-} mice exhibit dramatically lower levels of pro-inflammatory cytokine expression in the brain and are protected from development of autoimmune encephalomyelitis [17].

We recently reported that in addition to macrophages and adipocytes, FABP4 is expressed in a subset of endothelial cells (ECs) in several normal tissues [18]. FABP4 expression is detected primarily in microvascular and small vascular ECs as well as in infantile hemangioma ECs, suggesting an association with an angiogenic phenotype. Consistent with these observations, we found that FABP4 levels are induced with the pro-angiogenic mediators, VEGF-A and bFGF in ECs. Furthermore, EC proliferation is significantly inhibited by short-hairpin RNA (shRNA)-mediated knockdown of FABP4. These data prompted us to investigate whether FABP4 could play a previously unrecognized pro-angiogenic role in ECs. The experiments presented in this paper identify a role for EC-FABP4 as a mediator of angiogenesis for the first time and provide novel insights into the mechanisms by which FABP4 modulates the angiogenic responses of ECs.

Materials and methods

Cell culture and reagents

Discarded human umbilical cord specimens were collected from Brigham and Women's Hospital with approval from the Institutional Review Board. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords according to the method described by Jaffe et al. [19] and cultured on 1 % gelatin-coated dishes in M199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10 % fetal bovine serum (FBS) (Invitrogen), 30 µg/ml endothelial cell growth factor supplement (ECGF) (BD Biosciences, Bedford, MA), 1 % penicillin/streptomycin (Invitrogen), and 0.1 % heparin (Sigma, St. Louis, MO, USA). HUVEC starvation medium contained 2 % FBS and no ECGF. Second- or third-passage HUVECs were used in all experiments. HEK-293T cells (ATCC, Rockville, MD, USA) were cultured in DMEM (Invitrogen) supplemented with 10 % FBS and 1× non-essential amino acids (Sigma). VEGF-A 165 (VEGF), mouse basic FGF (bFGF), and recombinant stem cell factor (SCF) were purchased from R&D Systems (Minneapolis, MN, USA). LY294002, Wortmannin, SB203580, L-NAME, and Rapamycin were purchased from Calbiochem (San Diego, CA, USA). Hydroxyurea was purchased from Sigma and Matrigel from BD Biosciences. SCF measurements were performed using an ELISA kit from R&D Systems.

RNA interference

RNA interference was performed using MISSION® TRC shRNAs (Sigma) targeting fatty acid binding protein 4 (SHGLY-NM-001442), Raptor (SHCLNG-NM-020761) and firefly luciferase (SHC007) as previously described [18].

RNA isolation, quantitative analysis of mRNA and endothelial cell biology specific microarray

—Total RNA was isolated from cells with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and first-strand cDNA was synthesized with the Superscript II First-Strand Synthesis System (Invitrogen). Endothelial Cell Biology PCR array (SABiosciences, Frederick, MD, USA) was performed using the manufacturer's instructions to compare the expression levels of 84 genes in VEGF-stimulated FABP4-knock-down (FABP4-KD) and control HUVECs. SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was employed for real-time PCR analysis. Cyclophilin A was used as an internal reference to normalize the target transcripts by the $2^{-\Delta\Delta CT}$ method [20]. The lowest value for each gene was assigned an arbitrary level of 1. Real-time PCR primers were designed using the IDT DNA software (www.idtdna.com; supplemental data, Table 1).

Immunoblot analysis

Cells were lysed in buffer containing 0.5 % TritonX100, 100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl pH 7.4, Phosphatase Inhibitor Cocktail Set II (Calbiochem) and the protease

inhibitor cocktail tablet (Roche, Indianapolis, IN, USA). Immunoblot analysis was performed as described previously [21]. All primary antibody incubations were performed at 4 °C overnight at the following dilutions: rabbit polyclonal anti-FABP4 (Abcam, Cambridge, MA, USA), 1:2000; rabbit polyclonal anti-Caspase-3 (Cell Signaling Technology, Beverly, MA, USA), 1:1000; rabbit monoclonal anti-Akt, 1:1000 (Cell Signaling), rabbit monoclonal anti-phospho-Akt (Ser473, Cell Signaling), 1:1000; rabbit polyclonal anti-c-kit (DAKO), 1:500; rabbit monoclonal anti-c-kit (Tyr703, Cell Signaling), 1:1000; monoclonal anti-eNOS (BD Biosciences), 1:2500; rabbit polyclonal phospho-eNOS (Ser1177, Cell Signaling), 1:1000; rabbit polyclonal p38 MAP kinase (Cell Signaling), 1:1000; phospho-p38 MAP kinase (Thr180/Tyr182, Cell Signaling), 1:1000; rabbit polyclonal Erk 1/2 (Cell Signaling), 1:1000; and phospho-Erk 1/2 (Thr202/Tyr204, Cell Signaling), 1:1000.

Cell survival and proliferation assays

Apoptosis was induced in HUVECs by serum deprivation (5 % FBS). After 24 h, floating and adherent cells were collected, stained with fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) (Biovision Inc, Mountain View, CA, USA), and analyzed by flow cytometry. In parallel experiments, apoptosis was assessed in HUVEC lysates using immunoblot analysis for total and activated caspase 3. Cell proliferation was measured using BrdU incorporation as previously described [18].

Migration assays

Chemotaxis/directed migration assay was performed using polycarbonate filter wells (transwell, 8- μ m pores; Coaster, Corning, NY, USA) coated with gelatin or matrigel. HUVECs were growth arrested by addition of 2 mmol/l hydroxyurea to the medium, then plated in the upper chamber in 0.1 % FBS, ECGF-free medium at a density of 1×10^5 cells per well. Transwell migration of ECs was stimulated by addition of VEGF (50 ng/ml) or 10 % FBS to the culture medium in the lower well. After 6 h, the upper surface of the insert was swabbed to remove non-migrating cells. Inserts were washed with PBS, fixed in 4 % formaldehyde and stained with Diff-Quick. Endothelial cell migration was quantified by counting the number of cells in three random fields ($\times 100$ total magnification) per insert.

Cellular wound assay was performed in a 6-well plate using 80–90 % confluent HUVECs. Three vertical scratches were made across each well with a flat-edge forceps. A horizontal reference line was drawn to denote the scratch field of view (FOV) at the scratch intersection. Images of the wounded cells were taken above and below the reference line at the scratch/cell interfaces at $t = 0$ and $t = 8$ h. The average scratch width was determined for each FOV and the distance migrated was calculated [22, 23].

Aortic ring assay

FABP4^{-/-} mice were backcrossed more than 10 generations into C57BL/6J genetic background as previously described [10]. The Harvard Medical Area Standing Committee on Animals approved this study. Abdominal aortas were harvested from 4 to 5 week-old FABP4^{-/-} or wild-type (WT) mice. 1-mm-long mouse aortic rings were embedded in Matrigel in 96-well plates and cultured in medium supplemented with bFGF for up to 7 days as previously described [24, 25]. Neovessel outgrowth was imaged using a Nikon D5-5 M camera (Nikon, Tokyo, Japan) and quantified using NIH Image J software.

Adenoviral infection of aortic explants

Adenoviral recombinant plasmids that harbor the FABP4 (Ad/FABP4) and control LacZ (Ad/LacZ) genes were prepared using the ViraPower Adenoviral Expression System

(Invitrogen) as previously described [26]. Adenoviruses were added to the culture medium of aortic rings for 24 h at a multiplicity of infection (MOI) of 10 in 96-well plates prior to embedding in Matrigel.

Capillary network formation

HUVECs were plated on Matrigel in 24-well plates at a density of 1×10^5 cells per well in 2 % FBS containing M199 and 30 $\mu\text{g/ml}$ ECGF, with or without SCF (100 ng/ml). Formation of capillary-like structures was monitored up to 8 h, at which time wells were photographed. Morphogenesis was quantified by measuring the length of capillary like structures per unit area.

Statistical analysis

All results are presented as mean $\pm$ SEM from a minimum of 3 independent experiments. Statistical significance was determined with the non-parametric Mann–Whitney U test (two-tailed). p values < 0.05 were considered significant.

Results

FABP4-deficient endothelial cells demonstrate enhanced apoptosis

To determine whether FABP4 regulates the survival of ECs, the response of FABP4-KD HUVECs to serum deprivation-induced apoptosis was examined. As we have previously published, lentivirus-mediated delivery of FABP4 shRNA to HUVECs consistently resulted in a knockdown efficiency of greater than 80 % [18]. Flow cytometry analysis revealed that approximately 23 % of FABP4-KD HUVECs cultured in 5 % FBS-containing media were positive for annexin V and negative for PI (early apoptotic cell population) compared with 16 % of control cells ($p < 0.01$, Fig. 1a). The effect of FABP4 on EC survival was further examined by detection of cleaved caspase 3 as a marker of apoptosis by immunoblotting (Fig. 1b). Control and FABP4-KD HUVECs were cultured in 5 or 10 % FBS-containing media with or without VEGF for 24 h. Cells and supernatants were collected and analyzed by immunoblotting. Cleaved caspase 3 was not detectable in control HUVECs, but was present in FABP4-KD HUVECs cultured in 10 % FBS. As expected, cleaved caspase 3 was detectable in control HUVECs cultured in 5 % FBS and was markedly increased in FABP4-KD HUVECs compared with control cells ($p < 0.05$). Cleaved caspase 3 levels in FABP4-KD HUVECs cultured in 5 % FBS were decreased with VEGF treatment but continued to be higher than the control cells.

FABP4-deficient endothelial cells exhibit impaired migration and invasion

Endothelial cell migration plays a central role in angiogenesis. To determine whether FABP4 has an effect on EC migration, we assessed chemotactic and random migration of FABP4-KD and control HUVECs using the transwell migration and cellular wound assays, respectively (Fig. 2). These experiments were performed using growth-arrested cells to ensure that the effect of FABP4 on cell proliferation would not be a confounding factor. First, the effect of FABP4 on transwell migration of HUVECs towards two chemoattractants, VEGF and 10 % FBS, was investigated. The migration of FABP4-KD HUVECs was significantly reduced in comparison to control cells in response to both VEGF and 10 % FBS gradients across gelatin-coated membranes ($p < 0.01$, Fig. 2a). Similarly, the ability of FABP4-knockdown ECs to invade through Matrigel-coated membranes was significantly diminished compared to control cells (not shown). Random migration of FABP4-KD ECs was also significantly compromised as assessed by the cellular wound assay compared to the control cells ($p < 0.01$, Fig. 2b).

FABP4^{-/-} aorta demonstrates impaired angiogenic sprouting

To analyze the role of FABP4 during blood vessel sprouting, we next employed the aortic ring assay as an ex vivo model of angiogenesis. Aortic rings from 4 to 5 week-old, gender-matched FABP4^{-/-} and WT mice were embedded in Matrigel and supplemented with bFGF for 7 days as previously described [24]. While WT mouse aorta demonstrated a robust sprouting angiogenesis as expected, the number of sprouts and branches were significantly reduced in FABP4^{-/-} aortic rings (Fig. 3a, b). In reconstitution experiments, FABP4^{-/-} aortic rings were transduced with adenoviruses harboring lacz or FABP4 genes. This resulted in recovery of sprouting angiogenesis in ad/FABP4, but not ad/lacz transduced FABP4^{-/-} aortic rings.

FABP4 is a downstream target of mTORC1 in ECs

VEGF orchestrates EC functions that are essential for angiogenesis via multiple signaling pathways, including the Akt/PI3 kinase, eNOS, p38MAPK, and mTOR pathways. Having found a significant impact of FABP4 on VEGF-mediated EC survival and migration, we sought to identify VEGF-induced signaling pathway(s) that regulate FABP4 expression in ECs. For this purpose, we screened the effect of a panel of specific inhibitors of major signaling pathways on VEGF-induced expression of FABP4. HUVECs were incubated with VEGF and the inhibitors at varying doses for 48 h, and cell lysates were analyzed for FABP4 expression by immunoblotting (Fig. 4a). The inhibitors that were tested included LY294002 (PI3K inhibitor), Wortmannin (PI3K inhibitor), SB203580 (P38 inhibitor), L-NAME (eNOS inhibitor) and rapamycin (mTOR inhibitor). VEGF-induction of FABP4 protein levels was not decreased in HUVECs that were treated with LY294002, Wortmannin, L-NAME, or SB203580. In contrast, FABP4 expression was significantly reduced in HUVECs treated with rapamycin, thus suggesting that FABP4 is regulated by the mTOR complex 1 (mTORC1) pathway in ECs. To confirm the role of mTORC1 in regulation of FABP4, HUVECs were transduced with lentiviruses encoding shRNA targeting Raptor (raptor-shRNA), one of the three components of the mTORC1 complex, or a control shRNA (Fig. 4b). The efficiency of Raptor-knockdown (Raptor-KD) was confirmed by immunoblotting for phospho-S6 (pS6), a marker of mTORC1 activation. Consistent with the rapamycin data, decreased activation of mTORC1 by Raptor-KD resulted in a marked decrease in FABP4 expression levels both under baseline conditions and in response to VEGF treatment.

FABP4 modulates the expression of genes that have significant roles in angiogenesis

To begin to understand the molecular mechanisms that mediate the pro-angiogenic effects of FABP4 in ECs, we stimulated FABP4-KD and control HUVECs with VEGF for 12 h, then screened the mRNA expression levels of 84 genes that play important roles in EC biology using a targeted PCR array. In this initial analysis, 61 genes revealed critical threshold (CT) values of less than 38, our arbitrary cut-off, in control cells (Table S2 in the online supplement). We then focused on a subset of genes with known important functions in angiogenesis that also demonstrated a greater than 40 % alteration in their expression levels on this initial screen and analyzed their mRNA expression levels in FABP4-KD and control HUVECs both under baseline conditions and following VEGF-stimulation by real-time PCR (Table 1). There were significant decreases in the mRNA expression levels of eNOS, ICAM1 and selectin L in FABP4-KD HUVECs compared with controls both under baseline and VEGF-stimulated conditions, whereas the mRNA levels of integrin alpha 5 (ITGA5) and integrin beta 3 (ITGB3) were significantly decreased only in VEGF-stimulated FABP4-deficient cells compared to controls. An interesting and novel alteration was observed in the expression levels of c-kit, a tyrosine kinase receptor. C-kit was not regulated by VEGF, but c-kit mRNA levels were markedly increased in FABP4-KD cells. This finding prompted us to evaluate the effect of FABP4 on expression levels of the c-kit ligand, stem cell factor

(SCF). FABP4 deficiency led to an ~85 % decrease in SCF mRNA levels in both vehicle- and VEGF-stimulated HUVECs.

Effect of FABP4 on VEGF- and SCF-induced signaling pathways

To determine whether FABP4 modulated the activation of VEGF-induced signaling pathways, FABP4-KD and control HUVECs were stimulated with VEGF, and cells were harvested at 0, 5, 15, 30, and 60 min following stimulation. Cell lysates were analyzed for total and phosphorylated forms of p38, Erk1/2, Akt and eNOS by immunoblotting (Fig. 5a). There were no significant alterations in total p38, ERK 1/2 or AKT levels, whereas phospho-p38 levels were significantly diminished and phospho-AKT levels were increased in FABP4-deficient HUVECs compared with control cells (densitometry is shown in Fig. S1). Consistent with the mRNA data, FABP4 knockdown led to a dramatic reduction in total and phosphorylated eNOS levels. We next investigated the activation status of c-kit in response to SCF (Fig. 5b). In accord with the mRNA data, total c-kit levels were dramatically increased in FABP4-KD ECs and as expected, SCF stimulation resulted in markedly higher levels of phosphorylated c-kit in FABP4-KD HUVECs compared to control cells.

Role of SCF/c-kit signaling pathway in impaired angiogenic activity of FABP4-deficient endothelial cells

To determine whether c-kit upregulation in FABP4-KD HUVECs could be secondary to decreased levels of its ligand, SCF, control and FABP4-KD HUVECs were cultured in the presence of SCF for 24 h, and c-kit levels were analyzed by immunoblotting (Fig. 6a). Treatment of FABP4-KD cells with exogenous SCF led to a significant decrease in c-kit levels, thus suggesting that the increased c-kit levels in these cells are, at least in part, secondary to decreased levels of SCF. To confirm the impact of FABP4 on autocrine SCF/c-kit signaling, we measured the levels of soluble SCF in conditioned medium of FABP4-KD and control HUVECs and found significantly reduced SCF levels in conditioned medium of FABP4-deficient HUVECs compared with control cells (13.3 ± 0.5 vs 5.8 ± 1.9 pg per 1×10^5 cells, $p < 0.01$) (Fig. 6b).

Having found a novel effect of EC-FABP4 on regulation of the SCF/c-kit signaling pathway, we next assessed the contribution of this pathway to the anti-angiogenic effects associated with FABP4 deficiency. Supplementation of culture media with exogenous SCF (100 ng/ml) resulted in a significant recovery in EC proliferation, apoptosis, and capillary network formation in FABP4-KD HUVECs compared with control cells (Fig. 6c–e; Fig. S2). SCF treatment also led to significantly increased angiogenic sprouting in FABP4^{-/-} aortic rings (Fig. 6f). Of note, exogenous SCF did not enhance the angiogenic capacity of control HUVECs in these experiments.

Discussion

Although the role of FABP4 in adipocytes and macrophages has been extensively investigated, FABP4 was only recently discovered in vascular ECs and hence its function(s) in this cell type is not known [18]. In this study, we identified EC-FABP4, for the first time, as an important regulator of angiogenesis-related functions, including EC survival, migration, and angiogenic sprouting. We also found that EC-FABP4 is strongly regulated by the mTORC1 pathway and its inhibitor rapamycin. Furthermore, we identified several key angiogenic signaling pathways that are modulated by FABP4 in ECs, including p38, eNOS, and SCF/c-kit. Finally, we demonstrated that provision of SCF to FABP4-deficient ECs significantly recovers the impaired angiogenic activity of these cells, thus identifying SCF/c-kit pathway as a major mediator of FABP4-induced pro-angiogenic activity.

Increased susceptibility of ECs to apoptosis is a limiting factor in angiogenesis and can actively lead to vessel regression in postnatal neovascularization [27]. VEGF inhibits EC apoptosis via transduction of multiple signaling pathways [28]. Remarkably, we observed that FABP4 deficiency alone was sufficient to induce apoptosis in HUVECs under baseline culture conditions. In response to serum starvation, a widely used model of apoptosis in ECs, FABP4-deficient HUVECs exhibited markedly increased apoptosis compared to control cells that could only be partially rescued with VEGF. Previously, we had found a similar effect of VEGF on attenuated proliferation of FABP4-deficient HUVECs and human dermal microvascular ECs [18]. We had also reported that FABP4 is induced by VEGF via VEGFR2. In addition to decreased survival, FABP4-deficient HUVECs demonstrated reduced migration and tube formation, an in vitro model of migration as well as morphogenesis, which are other essential stages in angiogenesis that are regulated by VEGF. Thus, FABP4 appears to be an important downstream mediator of VEGF/VEGFR2 in ECs and the absence of FABP4 significantly impairs VEGF-induced angiogenic functions. In contrast to HUVECs, FABP4 knockdown in primary human lymphatic ECs results in enhanced proliferation and tube formation (C. Yu and S. Cataltepe, unpublished observations). These observations support the specificity of our data for vascular ECs.

To identify pathways that could potentially link VEGF/VEGFR2 signaling to FABP4, we screened a number of inhibitors that target major signaling pathways that are downstream of VEGF. This screen provided evidence for the first time that FABP4 is regulated by the mTORC1 pathway in ECs. The mammalian target of rapamycin, mTOR, is a serine/threonine kinase that exists in mammalian cells as two distinct protein complexes, mTORC1 and mTORC2 [29]. mTORC1 is composed of mTOR, mLST8, and Raptor, and is highly sensitive to rapamycin. The mTOR pathway has been shown to promote EC survival and proliferation [30, 31] and its inhibitor rapamycin inhibits early angiogenic responses to VEGF primarily via inhibition of S6K1 signaling downstream of mTOR [32]. In our studies, both rapamycin and raptor knock-down significantly inhibited both baseline and VEGF-induced expression of FABP4 in HUVECs. These results suggest that induction of FABP4 by mTORC1 may be one of the mechanisms that underlies the angiogenic effects of this pathway and hence, the anti-angiogenic effects of rapamycin may be mediated, at least in part, by inhibition of FABP4 expression. Interestingly, similar to the functional assays, VEGF could still induce FABP4 expression in Raptor-deficient HUVECs, but the level of induction was lower than the control cells. This finding suggests that mTORC1 may not be the only signaling pathway downstream of VEGF that regulates FABP4 expression. The inhibitor studies have ruled out contribution of Akt/PI3 kinase, eNOS, and p38MAPK pathways to this process.

Our experiments also identified regulation of several key intracellular signaling pathways by EC-FABP4. For example, eNOS expression and hence eNOS activation was dramatically decreased in FABP4-deficient ECs. Activation of eNOS by VEGF has been shown to induce airway angiogenesis and overexpression of eNOS is associated with increased angiogenesis in the acute limb ischemia model [33, 34]. These findings suggest that decreased activation of eNOS may be one of the potential mechanisms of decreased angiogenic activity in FABP4-deficient HUVECs. In addition to angiogenesis, eNOS regulates several other important vascular responses. For example, a relative deficiency of bioavailable vascular NO increases risk of pathologies such as thrombosis, inflammation, neointimal proliferation, and vasoconstriction, ultimately leading to the development of atherosclerosis [35]. Thus, regulation of eNOS expression by FABP4 may have important implications for other vascular responses in addition to angiogenesis. Other FABP4-regulated molecules in HUVECs that have been implicated in angiogenesis include ICAM1 [36, 37], Selectin L [38], ITGA5 and ITGB3 [39, 40]. The functional significance of decreased expression of these mediators in FABP4-deficient ECs is not known at this time.

In this study, we primarily focused on investigation of the role of SCF/c-kit pathway on decreased angiogenic capacity of FABP4-deficient ECs as the most dramatic alteration in gene expression patterns in FABP4-deficient ECs affected this pathway. We found a marked increase in c-kit levels and a parallel decrease in SCF levels in FABP4-deficient HUVECs. The increased c-kit levels were primarily due to the diminished autocrine signaling by SCF, because SCF levels were decreased in the conditioned media and treatment with exogenous SCF significantly decreased c-kit expression in FABP4-deficient ECs. The role of SCF/c-kit signaling pathway in ECs is not well characterized, but previous studies have shown up-regulation of SCF and down-regulation of c-kit with inflammatory stimuli in ECs [41–43]. SCF has also been shown to promote survival, migration, and capillary tube formation of HUVECs [44, 45], although these findings were not reproducible in another study [46]. In our experiments, exogenous SCF did not enhance the angiogenic function of control HUVECs under baseline conditions. However, exogenous SCF led to a significant recovery in proliferation and tube formation of FABP4-deficient cells and angiogenic sprouting of FABP4^{-/-} aortic rings. Thus, disruption of SCF/c-kit signaling by FABP4 deficiency played a critical role in diminished angiogenic responses of FABP4-deficient ECs. In addition to its autocrine function in ECs, SCF supports proliferation and differentiation of human hematopoietic progenitors and plays a role in recruitment of endothelial progenitor cells [43, 47]. Thus it is conceivable that decreased SCF/c-kit signaling in FABP4^{-/-} ECs can lead to decreased vasculogenesis as well as angiogenesis. The mechanism by which FABP4 regulates SCF or eNOS expression is not known at this time, but likely involves transcriptional mechanisms that are currently under investigation.

In summary, our studies have unraveled a novel pro-angiogenic function for FABP4 in ECs. Endothelial cell-FABP4, which is regulated by VEGF and mTORC1, has an impact on activation of several mitogenic pathways and expression of several key mediators of angiogenesis (Fig. 7). The autocrine SCF/c-kit signaling plays a key role in mediating the pro-angiogenic effects of FABP4 in ECs. Collectively, our data suggest that FABP4 inhibition should be explored further as a potential therapeutic strategy for pathological angiogenesis. In this regard, the restricted expression pattern of FABP4, which contrasts with that of VEGF, a survival factor for many different cell types [48], may prove to be advantageous.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

FABP4-deficient HUVECs demonstrate enhanced apoptosis. **a** HUVECs were transduced with lentiviruses harboring control- or FABP4-shRNA, then cultured in 5 % FBS-containing medium. After 24 h, cells were harvested, stained with FITC-conjugated annexin and propidium iodide, and analyzed by flow cytometry. Representative plots and mean results $\pm$ SEM from 6 independent experiments are shown. **b** FABP4-deficient (F) and control (C) HUVECs were cultured in 10 % or 5 % FBS-containing media with or without VEGF for 24 h. Cells were harvested and immunoblotting for caspase 3 was performed. A *representative immunoblot* and a *bar graph* depicting mean densitometry results of cleaved caspase 3 (cl.cas3) levels normalized to uncleaved caspase 3 (uncl.cas3) levels from 4 independent experiments are shown. *Error bars* represent SEM

Fig. 2.

FABP4 promotes migration and invasion of HUVECs. **a** Representative images of filters stained with Diff-Quick in a transwell cell migration assay. FABP4-KD or control HUVECs were plated in the upper chamber of a transwell at a density of 1×10^5 cells in 0.1 % FBS, ECGF-free medium and transwell migration was stimulated by addition of VEGF (50 ng/ml) or 10 % FBS to the media in the lower well. After 6 h, the upper surface of the insert was swabbed to remove non-migrating cells. Inserts were washed with PBS, fixed in 4 % formaldehyde and stained with Diff-Quick. Endothelial cell migration was quantified by counting the number of cells in three random fields (9100 total magnification) per insert. *Bar graph* represents mean $\pm$ SEM from 3 independent experiments. *Scale bar* 100 μ m. **b** Representative images of a cellular-wound assay immediately and 8 h after wounding. Control and FABP4-deficient HUVECs were grown to 80–90 % confluency in 6-well plates. Three vertical scratches were made across each well with a flat-edge forceps. A horizontal reference line (shown in *blue color*) was drawn to denote the scratch field of view (FOV) at the scratch intersection. Images of the wounded cells were taken above and below the reference line (*black arrow*) at the scratch/cell interfaces at t = 0 and t = 8 h. The average scratch width was determined for each FOV and the distance migrated was calculated. *Bar graph* represents mean $\pm$ SEM from 4 independent experiments. *Scale bar* 100 μ m

Fig. 3. Angiogenic sprouting is impaired in FABP4^{-/-} mouse aortic ring explants. **a** 1-mm-long WT and FABP4^{-/-} mouse aortic rings were embedded in Matrigel in 96-well plates and cultured in medium supplemented with bFGF. In reconstitution experiments, FABP4^{-/-} mouse rings were transduced with ad/lacZ or ad/FABP4 for 24 h before embedding in Matrigel. Representative images are shown. **b** The number of angiogenic sprouts arising from the aortic rings and the number of branches arising from the sprouts was quantified. Bar graphs represent mean $\pm$ SEM, $n = 8-10$ mice per group. Scale bar 100 μ m

Fig. 4.

FABP4 expression is regulated by mTORC1 pathway in HUVECs. **a** HUVECs were treated with VEGF (50 ng/ml) in the presence of Rapamycin (mTOR inhibitor, 20 nM), SB203580 (P38 inhibitor, 5 μ M), L-NAME (eNOS inhibitor, 2 mM), Wortmannin (PI3K inhibitor, 100 nM), and LY294002 (PI3K inhibitor, 100 μ M) for 48 h and cell lysates were analyzed for FABP4 expression by immunoblotting. A *representative immunoblot* shows that Rapamycin treatment markedly reduces VEGF-induction of FABP4. **b** Raptor-knockdown was achieved by lentivirus-mediated transduction of HUVECs with short-hairpin RNA targeting Raptor and confirmed by immunoblotting for pS6, a marker of mTORC1 activation. A *representative immunoblot* shows markedly decreased FABP4 levels in Raptor-deficient cells under baseline conditions as well as with VEGF treatment

Fig. 5. FABP4-deficient HUVECs demonstrate impaired p38 signaling and decreased eNOS and c-kit expression. **a** FABP4-KD and control HUVECs were treated with VEGF (50 ng/ml) for the indicated times and cell lysates were analyzed by immunoblotting using antibodies against total and phosphorylated forms of the signaling molecules p38, ERK, AKT, and eNOS. A representative immunoblot is shown. Equal amounts of protein were loaded for both control and FABP4^{-/-} cells for each different target. **b** Phosphorylation of c-kit was analyzed by immunoblotting at the indicated times following treatment of FABP4-KD and control HUVECs with SCF (100 ng/ml)

Fig. 6.

SCF recovers attenuated angiogenic activity of FABP4-deficient HUVECs and FABP4^{-/-} aortic explants. **a** FABP4-knockdown (FABP4-KD) and control HUVECs were treated with SCF (100 ng/ml) for 24 h and cell lysates were analyzed for c-kit expression by immunoblotting. A representative immunoblot is shown. **b** Following lentiviral transductions and puromycin treatment for 24 h, FABP4-KD and control HUVECs were cultured for 48 h in 6-well plates. Cells were harvested and cell numbers were determined. The amount of SCF in the conditioned media was measured using an ELISA kit and normalized to total cell numbers. **c** BrdU incorporation of FABP4-KD and control HUVECs was measured following SCF or vehicle treatment for 24 h using an ELISA kit. Mean values $\pm$ SEM from 4 independent experiments is shown. **d** Apoptosis was induced in 5 % FBS-containing medium with or without SCF, and cell lysates were analyzed for caspase 3 activation. A representative immunoblot from 3 independent experiments is shown. **e** FABP4-KD and control HUVECs were plated on Matrigel in 24-well plates in culture medium with or without SCF (100 ng/ml). Formation of capillary-like structures was monitored and photographed at 8 h. Morphogenesis was quantified by measuring the length of capillary like structures per unit area. Bar graph represents mean values $\pm$ SEM of 3 independent experiments. **f** Aortic rings from WT and FABP4^{-/-} mice were embedded in matrigel and cultured in medium containing SCF or vehicle for 7 days. The number of sprouts was quantified. Bar graph represents mean $\pm$ SEM, n = 5–11 mice per group

Fig. 7.

FABP4 regulates angiogenic capacity of endothelial cells. A schematic diagram of regulation and function of FABP4 in ECs is shown. *Dashed arrows* indicate hypothetical relationships that were not directly investigated in this study. Endothelial cell-FABP4 expression is induced by VEGF/VEGFR2 and mTORC1 pathways. FABP4 regulates the expression of several key genes that modulate angiogenic capacity of ECs likely at the transcriptional level

Table 1

Alterations in mRNA levels of select genes in control and FABP4-deficient HUVECs at baseline and in response to VEGF stimulation^a

Gene name (symbol)	Control	FABP4 ^{-/-} (mean ± SD)	P value	Control + VEGF (mean ± SD)	FABP4 ^{-/-} + VEGF (mean ± SD)	P value
Nitric oxide synthase 3 (eNOS)	1	0.51 ± 0.27	<0.01	2.21 ± 0.80	0.47 ± 0.27	<0.01
Intercellular adhesion molecule 1 (ICAM1)	1	0.47 ± 0.11	<0.01	2.62 ± 0.91	0.80 ± 0.18	<0.01
Selectin L (SELL)	1	0.22 ± 0.09	<0.01	1.28 ± 0.47	0.12 ± 0.06	<0.01
Selectin E (SELE)	1	0.95 ± 0.45	NS	5.14 ± 2.39	2.02 ± 0.78	NS ^b
Integrin, alpha 5 (ITGA5)	1	1.01 ± 0.44	NS	1.82 ± 0.76	0.97 ± 0.40	<0.05
Integrin, beta 3 (ITGB3)	1	0.69 ± 0.34	NS	1.24 ± 0.33	0.50 ± 0.10	<0.01
c-KIT ^c	1	10.05 ± 4.83	<0.01	1.02 ± 0.44	8.61 ± 4.51	<0.01
Stem cell factor (SCF)	1	0.16 ± 0.03	<0.01	2.97 ± 1.15	0.30 ± 0.22	<0.01

^aRelative steady-state mRNA levels were determined by real-time PCR. Mean ± SEM is based on 3–5 replicate experiments using different batches of pooled HUVECs

^bNot significant

^cV-kit Hardy–Zuckerman 4 feline sarcoma viral oncogene homolog