4.1 Abstract

Pseudomyogenic hemangioendothelioma (PHE) is an extremely rare locally aggressive neoplasm with endothelial differentiation, which often presents with multiple lesions. These tumors have characteristic *SERPINE1-FOSB* fusions. We report a 17 years old patient with advanced unresectable PHE with a durable complete remission to the multi-tyrosine kinase inhibitor telatinib. The aim of this study was to generate an in vitro model for PHE, to study the functional consequences of *SERPINE1-FOSB* in endothelial cells, and its interaction with telatinib, to biologically substantiate the complete response to telatinib.

As the fusion results in overexpression of a truncated form of *FOSB*, we overexpressed truncated FOSB in normal endothelial cells.

Truncated FOSB significantly affected tumor growth in three-dimensional (3D) on matrigel with increased and sustained sprouting. Moreover, truncated FOSB acted as an active transcription factor capable to regulate its own transcription, as well as to upregulate *PDGFRA* and *FLT1* expression (four-fold). Telatinib decreased proliferation and tumor growth in 3D and induced apoptosis. As expected, telatinib blocked VEGF signaling as phosphorylation of ERK was abolished. Interestingly, in FOSB overexpressing cells, telatinib specifically affected PDGFRA, FLT1, and FLT4 signaling and downregulated SERPINE1, thereby affecting the self-regulation of the fusion gene.

We provide a biological substantiation of a complete clinical remission that was seen in a patient with PHE, showing that telatinib indirectly interferes with the self-regulated expression of the fusion product. Thus, telatinib or any other currently available VEGFR1-4/PDGFRA inhibitor could be a highly specific treatment option for patients with multifocal unresectable PHE.

4.2 Introduction

Pseudomyogenic hemangioendothelioma (PHE) is a locally aggressive and rarely metastasizing tumor, predominantly affecting young adults. It is an extremely rare entity that occurs more frequently in males than in females (41 vs. 9). Most PHE patients present with multifocal disease (33 of 50 patients) with multiple discontiguous nodules present in different tissue planes (1, 2). This multicentric appearance combined with its locally aggressive behavior can make PHE difficult to treat.

PHE was first described as "epithelioid sarcoma-like hemangioendothelioma" in 2003, which was based on the presence of large cells with abundant eosinophilic cytoplasm at microscopy with keratin positivity (3). In 2011, based on a series of 50 patients, the proposed terminology was changed into PHE based on the myogenic appearance combined

with the evidence of endothelial differentiation by IHC, as well as the lack of a relation with epithelioid sarcoma (2).

Characteristic for PHE is a translocation between chromosomes 7 and 19. This translocation was later found to involve *SERPINE1* and *FOSB*. The translocation leads to a swap of the 5'-UTR regions and a loss of the first 48 amino acids of the FOSB protein (4, 5). FOSB is a member of the Fos family of proteins.

Fos family members can bind to Jun proteins thereby forming heterodimers, which make up the Activator Protein 1 (AP-1) transcription factor. By regulating the function of several genes such as FLI1 and FAS, this transcription factor is involved in many cellular functions including proliferation, differentiation, and transformation (6, 7). Among vascular tumors there is often involvement of the Fos family members. In epithelioid hemangioma, another locally aggressive vascular tumor, FOS and FOSB were also described to be involved in a translocation (8–10).

Based on the observation of a durable complete response following prolonged exposure to telatinib, an orally available tyrosine kinase inhibitor targeting VEGFR, PDGFR, and KIT, in a 17 years old male who presented with advanced, unresectable PHE, we here aimed to elucidate the underlying molecular mechanism of response to telatinib in PHE. As there are no PHE cell lines available we first needed to establish a model for functional analysis of PHE. We opted to use normal endothelial cells, human umbilical vein endothelial cells (HUVEC), in which we overexpressed the truncated FOSB protein, that is, lacking the first 48 amino acids of the FOSB protein that are lost in PHE due to the *SERPINE1-FOSB* fusion. We subsequently use this model to investigate the effect of telatinib on PHE. Based on the complete response we observed in the patient, we hypothesized that telatinib has a direct effect on the function of the *SERPINE1-FOSB* fusion product, the tumor-driving event in this particular tumor type.

4.3 Patient and Methods

4.3.1 Patient

A 17 years old male, with no prior history of disease, presented with multiple skin lesions on the head and neck. Excisional biopsy of one of the lesions revealed a cellular proliferation of spindled and epithelioid cells, with abundant eosinophilic cytoplasm, that occupied the dermis and extended into the subcutis (figure 4.1a). The nuclei were round to oval with one or more prominent nucleoli. IHC revealed that the tumor cells were positive for CD31, vimentin, and keratin AE1AE3, whereas CD34, S100, CD68, HHV8, podoplanin (D2-40), melan A, CD56, CD57, EMA, smooth muscle actin, KL1, CAM5.2, CD45, keratin 20, CD30, actin (HHF35), and desmin were negative. The differential diagnosis

included epithelioid sarcoma and epithelioid sarcoma-like hemangioendothelioma/PHE, an entity that was just described at that time (2, 3). Based on the strong expression of CD31 the diagnosis of PHE was favored. As the patient also had enlarged lymph nodes in the cervical area, a fine needle aspiration was performed and tumor involvement of locoregional lymph nodes in the neck was confirmed. CT of the chest and abdomen showed no distant metastasis. Initially, the patient was treated with six rounds of docetaxel, which was chosen given the then recently described activity of taxanes in vascular sarcomas of the scalp (11). Docetaxel yielded a partial, although short-lasting response, followed by rapid progression of the disease in the head and neck area after three months. The disease was too extensive for an operation (figure 4.1e, left). An excisional biopsy of one of the lesions confirmed the lack of response to chemotherapy. Two months later, the patient was included in a multicentre phase I dose escalation study for telatinib (BAY 57-9352), an orally available, small-molecule multi-tyrosine kinase inhibitor (12), which was open for patients with advanced or metastatic solid tumors and for whom no standard therapy is available. The patient has given written informed consent for the use of material, and publication of clinical data including photographs.

4.3.2 Immunohistochemistry

FOSB and KIT IHC was performed using 4-μm-thick tissue sections. Paraffin was removed with xylene and sections were rehydrated in a gradient of ethanol. Endogenous peroxidase was blocked using 0.3% H_2O_2. Microwave antigen retrieval was performed in Tris-EDTA (pH 9.0). FOSB antibody (Cell Signaling) or KIT antibody (Dako) was incubated with the cells overnight at 4°C. Rabbit secondary antibody was used and detected with DAB (3,3'-diaminobenzidine). Counterstaining was performed with hematoxylin.

4.3.3 Fluorescence in situ hybridization

FISH has previously been described by our group (8). BAC probes were selected surrounding *FOSB* (CTB-14D10 and RP11-84C16) and *SERPINE1* (RP11-44M6 and RP5-1059M17). Four-micrometer-thick tissue sections were cut from the paraffin embedded excisional biopsy before chemotherapy. BAC probes were tested on metaphase control slides.

4.3.4 Polymerase chain reaction

RNA was isolated from fresh frozen tissue and cultured cells using the Direct-zol RNA Isolation Kit (Zymo research). cDNA was made using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed with SybrGreen (Bio-Rad) on a Bio-Rad CFX384

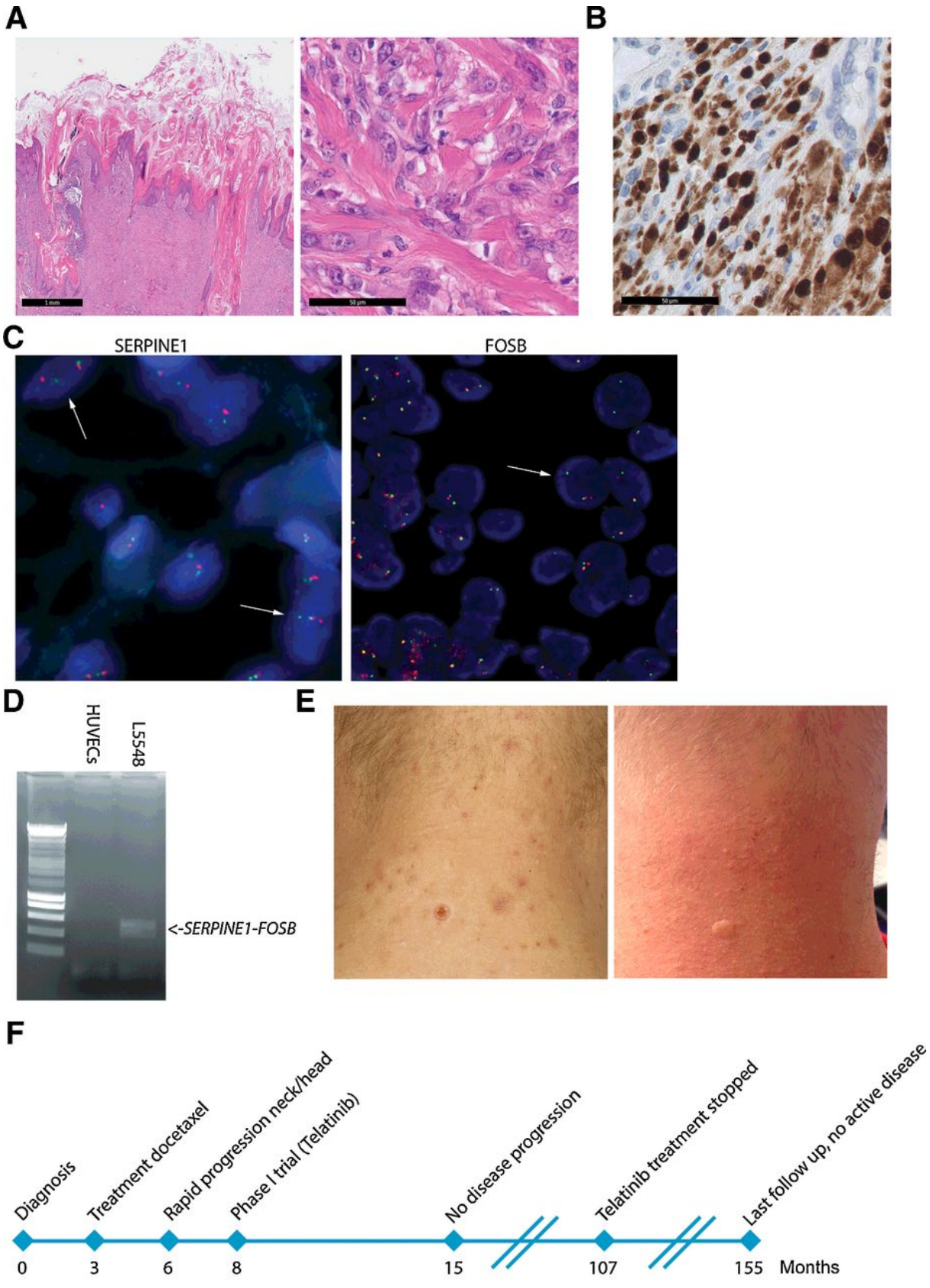

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Figure 4.1: Diagnosis of PHE was confirmed by detection of the *SERPINE1-FOSB* gene fusion in a tumor from a 17 years old patient with advanced inoperable PHE who was treated with telatinib and showed a complete remission. (a) Left panel shows a low power H&E image of the lesion showing that the lesion infiltrates the epidermis. The scale bar indicates 1 mm. Right panel shows a high power H&E showing tumor cells with a rhabdomyoblast-like appearance. The scale bar indicates 50 μm. (b) IHC confirms high expression of FOSB in the nuclei of the tumor cells. The scale bar indicates 50 μm. (c) Using FISH, the involvement of *SERPINE1* locus (left) and *FOSB* locus (right) was confirmed using split apart FISH probes (arrows indicate cells with split apart signals). (d) PCR on cDNA made from RNA of frozen patient tumor tissue (L5548) shows a distinct band indicating expression on RNA level of the fusion gene at the expected size. (e) Left panel shows a picture of the skin lesion as seen at the time of diagnosis. Multiple nodules with hyperkerastosis and central ulcerations were seen. Right panel is the same area 156 months after first diagnosis showing complete remission of the lesions. The picture shows that the hyperkeratotic lesions have disappeared. (f) Timeline shows the course of the disease, from the initial diagnosis to rapid progression while treated with docetaxel to the complete remission nine years after starting telatinib treatment.

Touch (Bio-Rad). For regular PCR Phusion proofreading enzyme was used (NEB). To visualize amplified DNA fragments were size separated on a 1% agarose gel. Used primers are listed (Supplementary Table S1 available online). All real-time PCR experiments were performed in triplicates.

4.3.5 Cell culture

Primary pooled HUVECs (Lonza) were cultured in EGM-2 medium (Lonza) on 0.2% gelatin coated culture dishes. Cells were serum starved with M199 medium (Gibco) for 6 hours before introducing EGM-2 or M199 medium supplemented with VEGF. Cells were checked for mycoplasma with the MycoAlert Kit (Lonza).

4.3.6 Plasmid and shRNA

Human *FOSB* and truncated *FOSB* were cloned in frame with a Flag tag into pLV lentiviral expression vector. ShRNA was selected from the Sigma Mission shRNA library. Knockdown efficiency of the selected shRNAs was tested with real-time qPCR (Supplementary Table S2 available online).

4.3.7 HUVEC tube formation assay, proliferation assay, and analysis

Tube formation assays were performed on 96-well plates coated with 60 μL matrigel (Lonza). Cells were seeded at a density of 20,000 cells per well in 200 μL EGM-2 medium. To induce tube formation, 50 ng/mL VEGF was added. Tube formation was analyzed with Stacks (in house developed software tool, Molecular Cell Biology, LUMC). To measure relative proliferation 11 μL PrestoBlue Cell Viability reagent (Thermo Fisher Scientific) was added to each well after which the cells were incubated for 30 minutes. Measurements were performed with the VICTOR Multilabel Plate Reader (PerkinElmer). As vehicle control, DMSO was added to the control cells at corresponding concentrations to exclude effects from the DMSO used to dissolve telatinib. All tube formation assays were performed in triplicates.

4.3.8 Chromatin immunoprecipitation

In short, chromatin immunoprecipitation (ChIP) was performed by crosslinking protein and DNA with a final concentration of 1% formaldehyde. Crosslinking was stopped with 1.25 M glycine. Chromatin was sonicated 15 minutes using a Bioruptor at 30 second intervals to generate 500 to 1,000 bp DNA fragments. Immunoprecipitation was performed with ProtA beads and monoclonal FOSB rabbit antibody (Cell Signaling). Thereafter DNA was eluted and quantitative real-time PCR was performed. Primers were designed to detect three different AP-1 binding sites near the *SERPINE1* promoter (Supplementary Table S3 available online). Targets for ChIP were identified with the DECODE database (SABioscience).

4.3.9 Antibodies, growth factors, and drugs

Western blotting and IHC have previously been described by our group (13). Antibodies were obtained from the following sources: Flag monoclonal rabbit antibody (F7425; Sigma); FOS monoclonal rabbit antibody (HPA018531; Protein Atlas); FOSB monoclonal rabbit antibody (#2251; Cell Signaling); phosphorylated ERK mouse monoclonal antibody (M8159; Sigma); JUN monoclonal rabbit (#9165; Cell Signaling); USP7 monoclonal rabbit (A300-033A; Bethyl); KIT polyclonal rabbit (A4502; Dako). Tube formation was stimulated with 50 ng/mL VEGF 165 (R&D systems). Cycloheximide was used to block translation at a concentration of 50 μg/mL (Sigma). Telatinib (Selleckchem) dissolved in DMSO was used at different concentrations.

4.3.10 Apoptosis and cell-cycle analysis

The Nucleocounter NC-250 (Chemometec) was used for apoptosis and cell-cycle analysis. For apoptosis, the cells were resuspended in PBS and stained with propidium iodide (PI) and VitaBright-48 (VB-48). For cell-cycle analysis the cells were fixed, permeabilized, and thereafter stained with DAPI. Analysis was performed with FlowJo (FlowJo, v10). Apoptotic cells were selected by gating for the PI and VB-48 negative cells.

4.4 Results

4.4.1 Detection of the *SERPINE1-FOSB* fusion confirms the diagnosis of PHE

We used IHC for FOSB, which was recently reported as a very specific marker for PHE (14), on the initial excisional biopsy and confirmed overexpression of FOSB in the nuclei of the tumor cells (figure 4.1b). Using FISH with split apart probes flanking *SERPINE1* and *FOSB* genes, we confirmed that both genes were involved in the fusion (figure 4.1c). Expression of the *SERPINE1-FOSB* fusion transcript was confirmed using RT-PCR performed on RNA isolated from fresh frozen tissue from the excisional biopsy (figure 4.1d).

4.4.2 Complete response after long-term telatinib treatment in a patient with pseudomyogenic hemangioendothelioma

Six weeks after starting telatinib treatment, one of the larger skin lesions was shed from the skin and disease progression had stopped. The treatment seemed to have a gradual, cyclic effect, with regular shedding of the skin lesions. The lymph nodes were small and non-palpable and therefore initially followed by CT but remained stable over the years. Therefore, it was decided to use visual inspection of the skin lesions to monitor disease activity. The patient had no side effects of the telatinib other than headache and treatment was continued. Another 4 years later, all skin lesions had disappeared and the disease was considered in complete remission (figure 4.1e). Nine years after first diagnosis, telatinib treatment was stopped (as it was no longer available) and the patient is still tumor free 4 years later after treatment cessation (figure 4.1f).

4.4.3 Overexpression of truncated *FOSB* in HUVECs as a model to study PHE

We overexpressed truncated *FOSB* (*FOSB*49-338) in HUVECs to generate a model to study PHE (figure 4.2a). We grew the HUVECs on matrigel to evaluate their behavior in 3D. We found that the HUVECs overexpressing truncated *FOSB* not only showed more tube formation compared to control HUVECs (Supplementary figure S1 available online), but also retained tube formation for more than 48 hours, where the pLV control cell network collapsed after 24 hours, which is normal for this cell type (figure 4.2b). 2D cell culture also showed a small difference in proliferation between truncated *FOSB* and pLV control HUVECs after 48 hours (P = 0.04; figure 4.2c).

4.4.4 Truncated FOSB acts as an active transcription factor capable of self-regulation

Next, we studied if the fusion affects the function, lifetime, and localization of FOSB. With immunoprecipitation we show that truncated FOSB forms a complex with JUN (figure 4.3a), showing that similar to normal FOSB, truncated FOSB still forms an AP-1 complex. Immunofluorescence showed that truncated FOSB, similar to wild-type FOSB, localizes to the nucleus (figure 4.3b). To see if the loss of the first 48 amino acids of FOSB affected its stability we treated the cells with cycloheximide, effectively blocking all translation. After blocking translation both the truncated and full length FOSB showed to be highly stable (figure 4.3c). To evaluate differences in downstream signaling, we evaluated expression of previously identified AP-1 targets as downstream markers of upregulated AP-1 signaling (13). With real-time qPCR we found that truncated *FOSB* upregulates *HEY1*, *JAG1*, *FAS* (respectively 3.18, 0.78, 0.98 log2 fold) and downregulates *VWF* and *ADAMTS13* (respectively -4.08, -1.53 log2 fold) compared with control HUVECs (figure 4.3d). To check the validity of our model we also investigated expression in the patient tumor tissue (which contains ∼60% tumor cells). *FOSB* was upregulated in both the patient biopsy and the HUVECs overexpressing truncated *FOSB* (respectively 8.68 and 13.99 log2 fold; Supplementary figure S2A available online). Similarly, in the tumor tissue from the patient, *HEY1*, *JAG1*, and *FAS* were upregulated (respectively 3.57, 1.51, and 3.31 log2 fold) and *VWF* was downregulated (-5.61 log2 fold) compared with normal HUVECs (Supplementary figure S2B available online). *ADAMTS13* was not detectable in the patient tumor biopsy. Thus, truncated *FOSB* functions as an active nuclear transcription factor in HUVECs regulating known AP-1 target genes. In the *SERPINE1* promoter we found three AP-1 consensus sites by searching in the DECODE (SABioscience) database (Chr7:100760761, Chr7:100761757, and Chr7:100766067 in hg18), indicating the AP-1

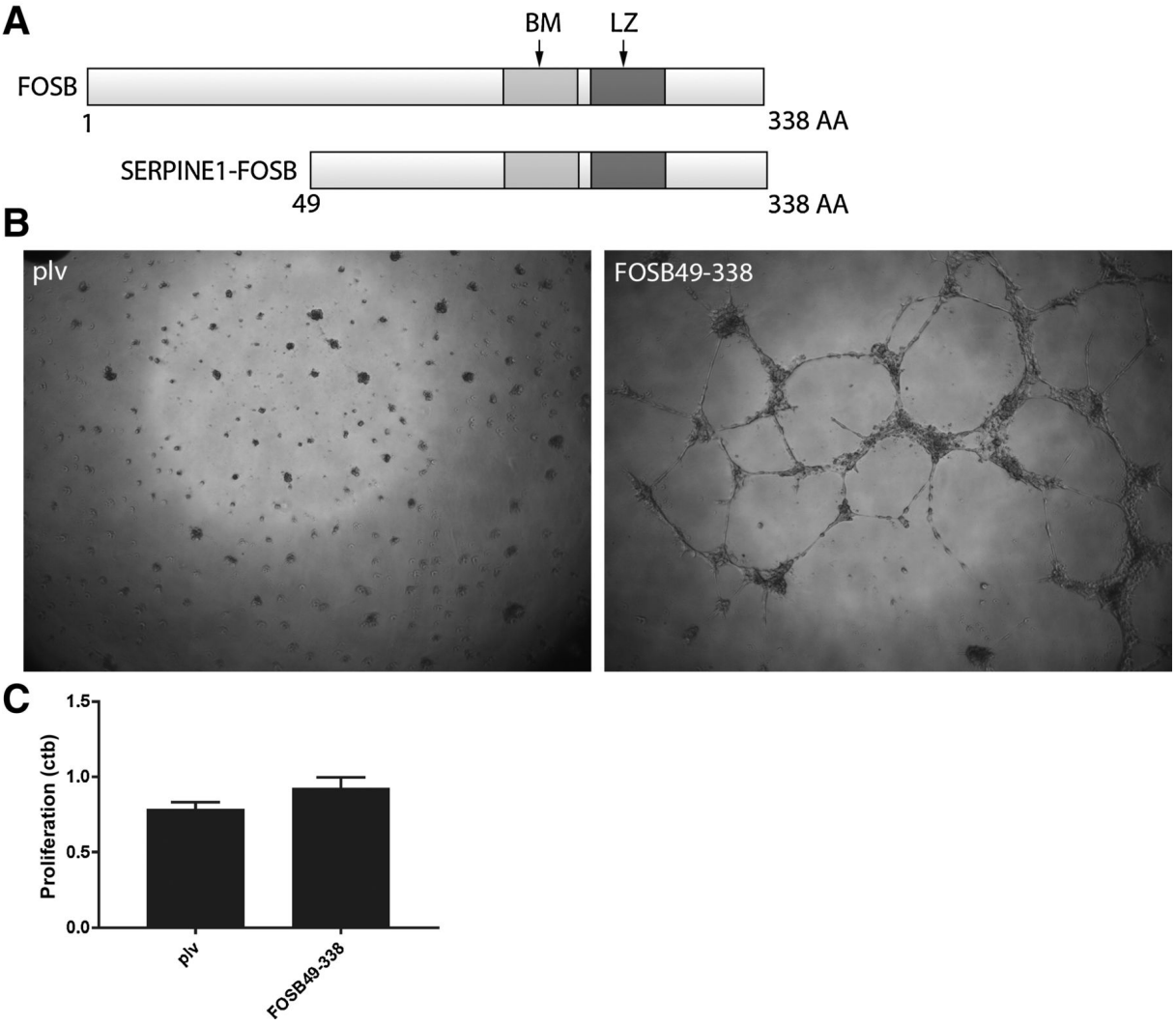

Figure 4.2: Truncated *FOSB* is representative of *SERPINE1-FOSB* and affects HUVEC growth in 2D and 3D with increased and sustained sprouting. (a) Schematic view of truncated FOSB protein. *SERPINE1* only retains its 5'-UTR whereas *FOSB* loses the coding region for the first 48 amino acids. The truncated FOSB retains the DNA Binding Motif (BM) and Leucine Zipper (LZ). (b) Tube formation after 48 hours. Control tube network (pLV) has collapsed while truncated FOSB overexpressing HUVECs retain the tube network showing that overexpression of truncated FOSB leads to increased and prolonged tube formation. (c) Presto Blue assay shows overexpression of truncated FOSB compared to pLV controls cells leads to an increase in proliferation ($P = 0.0429$).

transcription factor is an important regulator of *SERPINE1*. To investigate if truncated FOSB directly regulates *SERPINE1*, we first performed a chromatin pull-down with a FOSB antibody. With real-time qPCR for the AP-1 consensus sites in the *SERPINE1* promoter we detected an average enrichment of 2.18 log2 fold compared to the control cells (figure 4.3e). Furthermore, real-time qPCR shows an increase in *SERPINE1* expression (0.34 log2 fold) when truncated *FOSB* is expressed (figure 4.3f). Thus, it is likely that the *SERPINE1-FOSB* fusion is able to function as its own promoter and thereby regulates its own expression.

4.4.5 Telatinib blocks VEGFR downstream signaling and induces apoptosis in truncated FOSB overexpressing HUVECs

We assessed the response of HUVECs overexpressing truncated FOSB to the inhibitor. When growing HUVECs overexpressing truncated FOSB on matrigel, telatinib inhibits most tube formation at 5 μmol/L and completely abolishes tube formation at 10 μmol/L (figure 4.4a). To investigate if telatinib blocks signaling downstream of the VEGF receptors, we investigated the effect of telatinib on MAPK/ERK signaling. We found that telatinib reduces phosphorylation of KDR (VEGFR2) at 100 nmol/L and completely blocks phosphorylation at 2 μmol/L (figure 4.4b). Most phosphorylation of ERK is absent at 1 μmol/L and higher concentrations show no additional effect (figure 4.4c).

To see if telatinib leads to apoptosis (and not senescence or quiescence), we measured the cell cycle and the apoptotic fraction using an automatic fluorescence cell counter. We found no difference between the untreated and telatinib treated cells in the G2 fraction (from 8.97% in the untreated to 9.44% in the 10 μmol/L telatinib treated cells; supplementary figure S3 available online). The sub-G1 fraction however increases from 4.7% in the untreated to 10.7% in the 10 μmol/L telatinib treated cells. Furthermore, we found the fraction of apoptotic cells (negative for PI and VB-48) increases from 16.1% in the untreated to 26.5% in the 10 μmol/L telatinib treated cells (figure 4.4d).

4.4.6 Truncated FOSB sensitizes HUVECs to inhibition of the surface receptors FLT1 and PDGFRA by telatinib, leading to a reduced expression of the fusion product

Reportedly telatinib inhibits signaling through KIT, VEGFR, and PDGFR (15). To investigate which receptors are essential for growth of truncated FOSB overexpressing HUVECs in 3D, we selected shRNA targeting the aforementioned receptors. Normal HUVECs were sensitive to knockdown of *FLT4* (*VEGFR3*) and *KIT*. HUVECs overex-

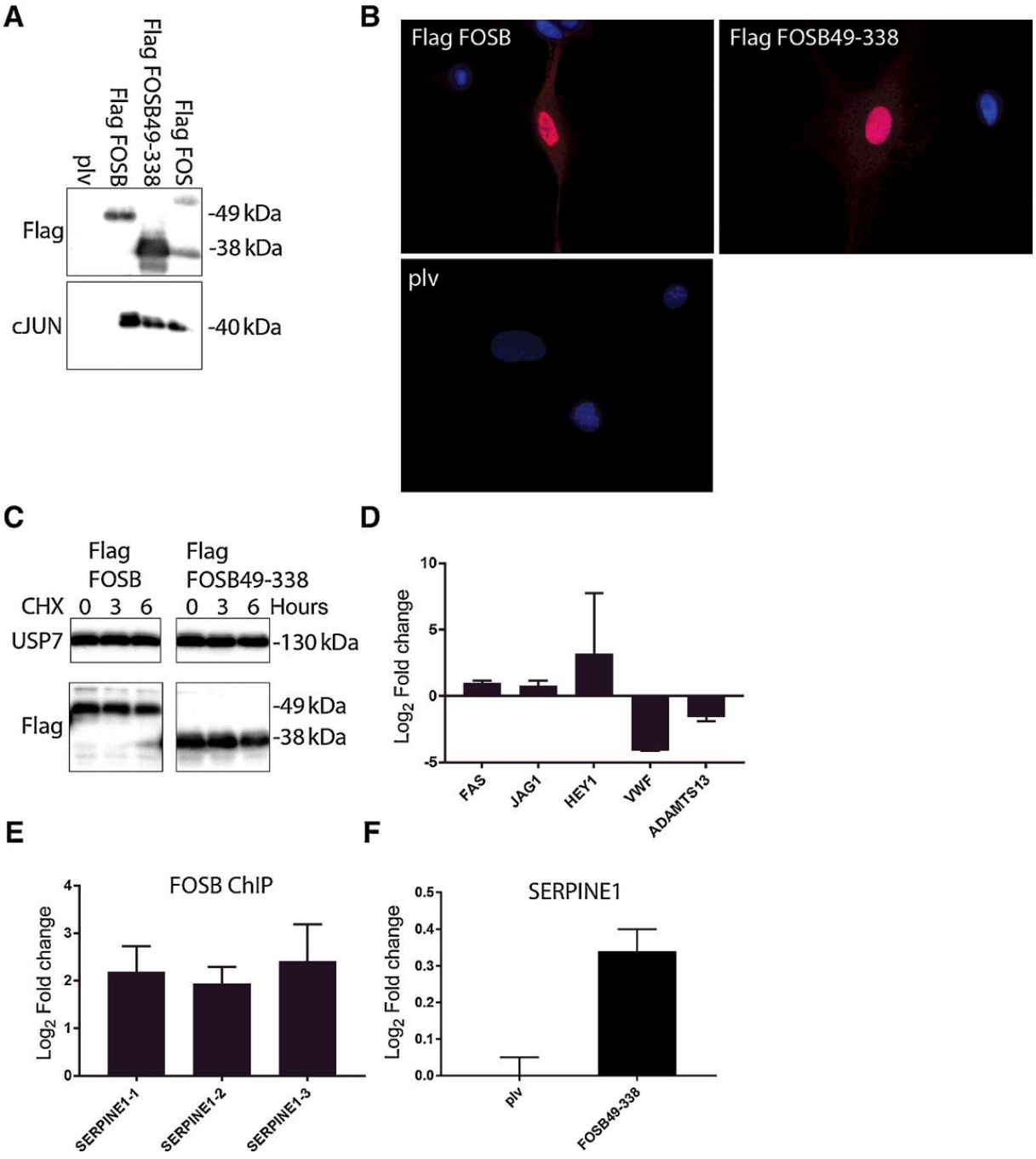

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Figure 4.3: Truncated FOSB still functions as a transcription factor in the AP-1 complex, localizes to the nucleus, and can regulate its own expression by acting on the *SERPINE1* promoter. (a) FOSB and truncated FOSB form heterodimers with JUN as seen when immunoprecipitating with Flag beads and subsequent detection of JUN suggesting they can form an AP-1 transcription factor. Flag tagged FOS was used as a positive control. (b) Immunofluorescence using anti-Flag antibody shows both FOSB and truncated FOSB (red) localize to the nucleus (blue) whereas no signal is detected in the pLV control cells. (c) HUVECs overexpressing FOSB and truncated FOSB were treated with Cycloheximide to see if the loss of the first 48 amino acids influences the turnover of the protein. No difference in lifetime was detected between the variants showing that the truncation does not affect the half-life of the protein and that both forms of FOSB proteins are active for over 6 hours. (d) Expression levels of the indicated transcripts in HUVECs overexpressing truncated FOSB compared to normal control HUVECs were determined by real-time qPCR showing upregulation of *FAS*, *JAG1*, and *HEY1*. *VWF* and *ADAMTS13* are downregulated. Truncated FOSB is therefore still capable of regulating downstream target genes. Expression was normalized against *HPRT1*. (e) All three AP-1 consensus sites in *SERPINE1* promoter were retained in the *SERPINE1-FOSB* fusion. With qPCR after ChIP we showed an enrichment for the promoter binding site when truncated FOSB was overexpressed. Immunoprecipitation was performed with a FOSB antibody. This indicates that *SERPINE1* expression is under direct control of truncated FOSB. (f) Overexpression of truncated FOSB upregulates SERPINE1 compared to control HUVECs as found with real-time qPCR for *SERPINE1* transcript. Expression was normalized against *HPRT1*.

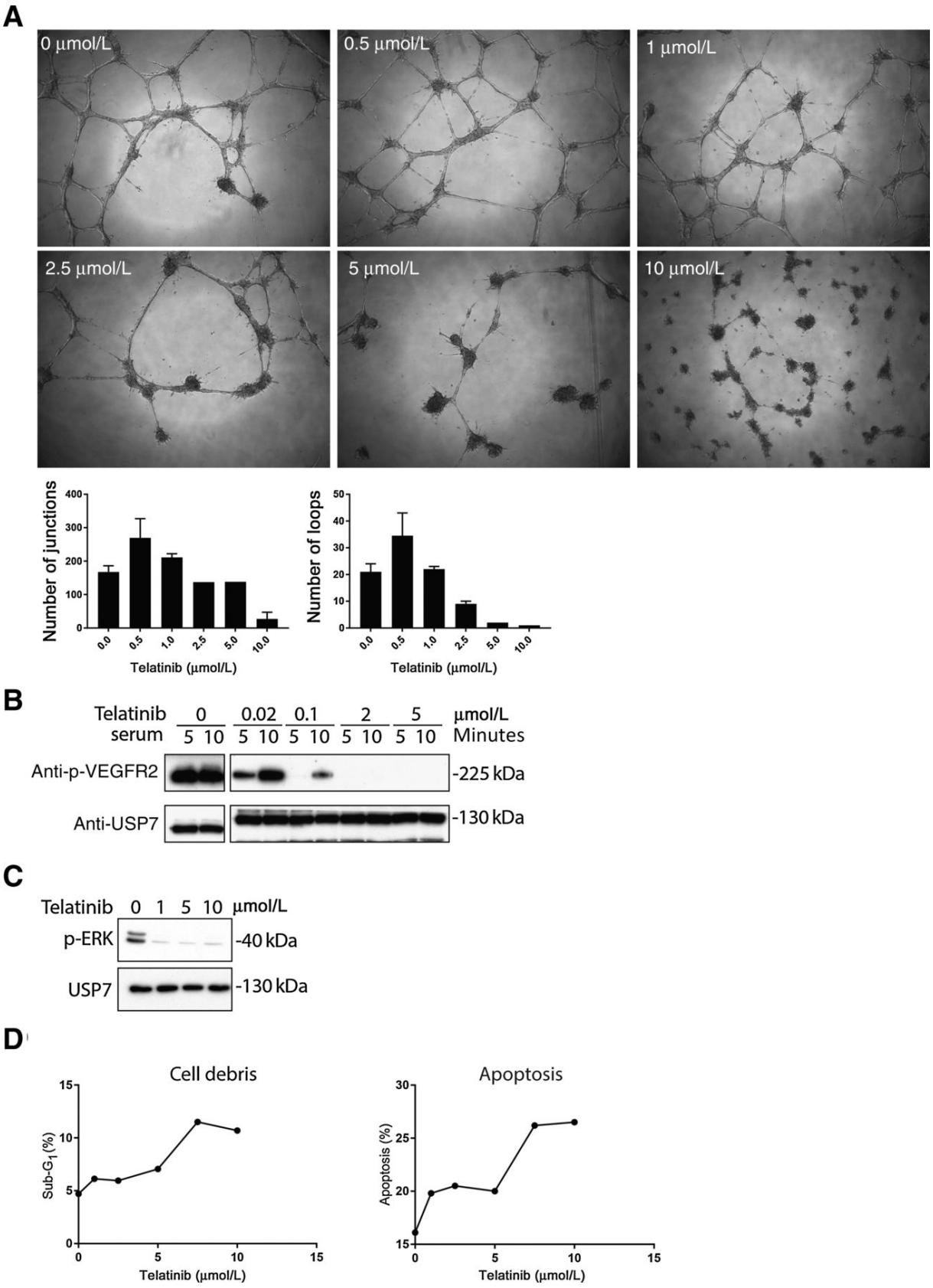

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Figure 4.4: Telatinib inhibits proliferation and tube formation through blocking VEGF receptor and its downstream signaling. (a) 3D assay on matrigel with HUVECs overexpressing truncated FOSB. Most tube formation is blocked at 5 μmol/L telatinib, whereas no tube formation is present at 10 μmol/L. Bottom part of the panel shows the decrease in calculated number of loops and junctions of the tubes with increasing doses of telatinib. (b) Serum starved and stimulated HUVECs overexpressing truncated FOSB were harvested 5 and 10 minutes after addition of serum. The cells were treated with telatinib at the indicated concentrations for 6 hours prior to addition of serum. At 100 nmol/L most phosphorylated VEGFR2 (KDR) is absent, whereas at 2 μmol/L it is completely absent. (c) HUVECs overexpressing truncated FOSB were treated with the indicated concentrations of telatinib 6 hours prior to addition of VEGF. Already at 1 μmol/L most phosphorylation of ERK is absent as detected with a phospho-ERK antibody detecting bands at 42 and 44 kDa, the 44 kDa band completely disappears whereas there is still a faint band visible at 42 kDa, which shows no further reduction at higher concentrations. (d) The left panel shows the sub-G1 cell fraction as found with the NC-250 nucleocounter, showing an increase in sub-G1 fraction with an increase in telatinib concentration. The right panel shows cell cycle and apoptosis as analyzed with the Nucleocounter NC-250. HUVECs overexpressing truncated FOSB were treated with telatinib at the indicated concentrations. An approximate two-fold increase of apoptosis was detected with the highest telatinib dose.

pressing truncated FOSB were also sensitive to knockdown of *FLT1* and *PDGFRA* as well as knockdown of *FLT4* (figure 4.5a; supplementary figure S4A available online). With real-time qPCR, we demonstrated that overexpression of truncated FOSB upregulates *FLT1* and *PDGFRA* (3.53 and 4.35 log2 fold, respectively; figure 4.5b). Thus, overexpression of truncated FOSB results in high expression of *FLT1* and *PDGFRA*, whereas its inhibition alters tumor growth in 3D, suggesting dependence. With the overexpression of truncated FOSB, *KIT* expression was reduced which is in line with the lack of KIT expression in the patient's tumor (supplementary figure S4B). The expression of *FLT1* and *PDGFRA* in the patients tumor, compared with normal HUVECs was 0.13 and 4.92 log2 fold, respectively (supplementary figure S2B).

As AP-1 is reportedly a downstream transcription factor of VEGF signaling (16), we hypothesized that inhibition of the VEGF receptors with telatinib would indirectly lead to a reduction in *SERPINE1* expression. We investigated this by serum starving and stimulating HUVECs which leads to an upregulation of SERPINE1. This upregulation could be blocked by treatment with telatinib (figure 4.5c), suggesting that blocking FLT1, FLT4, and PDGFRA with telatinib could lead to a reduction in expression of the *SERPINE1-FOSB* fusion protein.

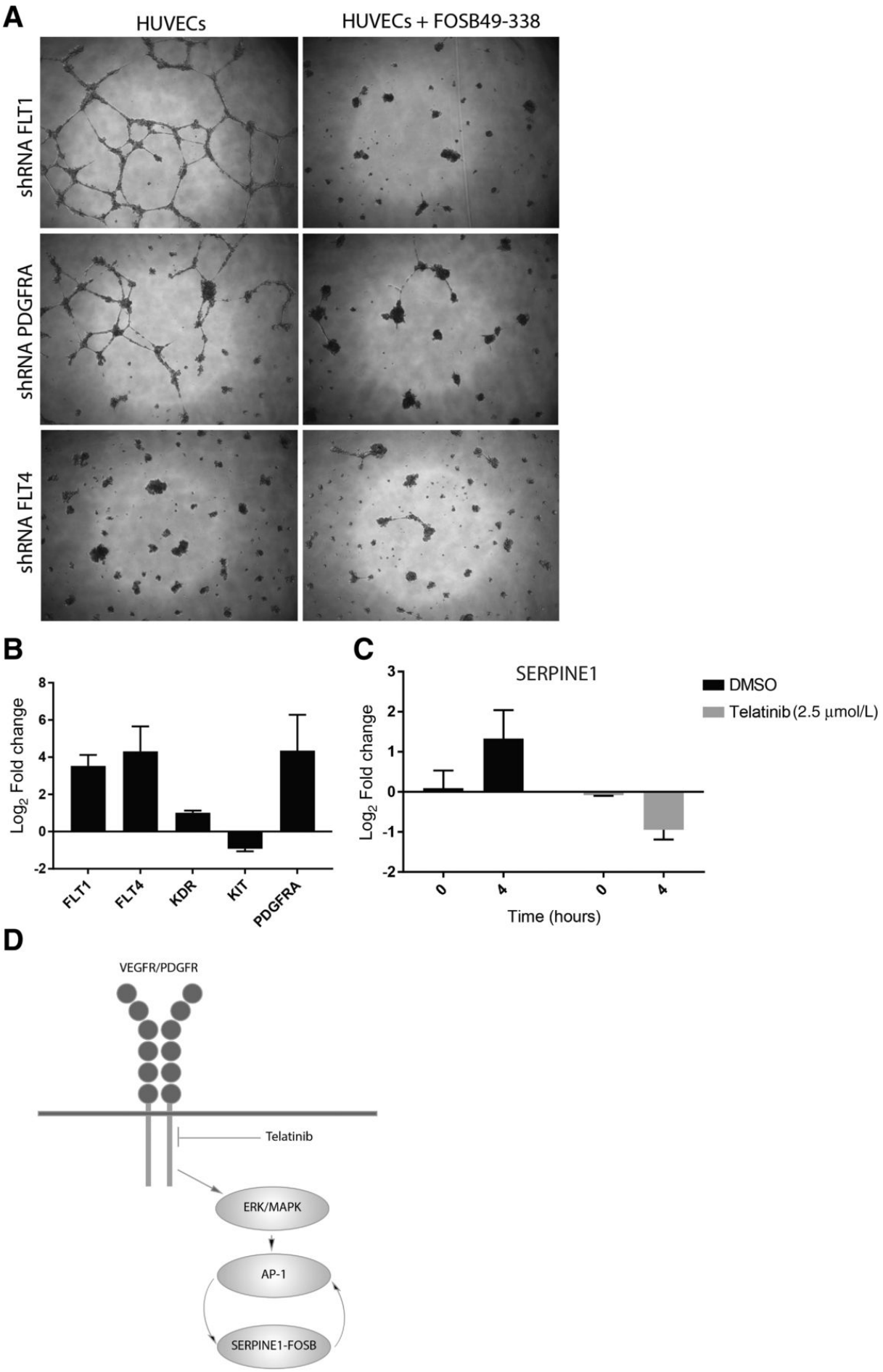

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Figure 4.5: Telatinib targets FLT1, FLT4, and PDGFRA, which are shown to be essential for tube formation in truncated FOSB expressing HUVECs. (a) 3D assay on matrigel with normal HUVECs and with HUVECs overexpressing truncated FOSB. ShRNA for *FLT4* efficiently blocks tube formation in both conditions whereas *FLT1* and *PDGFRA* knockdown only blocks tube formation when truncated FOSB is overexpressed. This suggests that truncated FOSB sensitizes the cells to *PDGFRA* knockdown. (b) Expression levels of the indicated transcripts in HUVECs overexpressing truncated FOSB compared with normal HUVECs were determined by real-time qPCR. *KIT* expression was reduced while *FLT1*, *FLT4*, and *PDGFRA* expression is increased by overexpression of truncated FOSB. Expression was normalized against *HPRT1*. (c) Serum starving and stimulating cells show an upregulation of the *SERPINE1* transcript as found with real-time qPCR. This upregulation is blocked when the HUVECs are treated with telatinib. Expression was normalized against *HPRT1*. (d) We propose a model for the modus of action for telatinib treatment in PHE. *SERPINE1-FOSB* can regulate its own expression while also being under control from the downstream signaling cascade of VEGFR and PDGFR surface receptors. Inhibition of these receptors reduces *SERPINE1-FOSB* expression, and expression is further diminished because self-regulation is reduced.

4.5 Discussion

PHE is a locally aggressive tumor of the so-called intermediate category in the WHO 2013 classification (17), which often presents with multiple discontiguous nodules. In this study we elucidated the biological effect seen in a patient who presented with advanced inoperable PHE, who was treated with telatinib and showed a durable complete response. We here present an in vitro model for PHE in which we show that truncated FOSB is capable of regulating *SERPINE1*, as well as being under control of AP-1 through signaling from VEGF- and PDGF-receptors yielding a positive feedback loop. We propose a model where telatinib inhibits FLT1, FLT4, and PDGFRA signaling, reducing initial expression of *SERPINE1-FOSB*. As there would be less *SERPINE1-FOSB* available, self-regulation is reduced which would further diminish the levels of *SERPINE1-FOSB* in the tumor cells. This effect, combined with essential signaling being blocked by inhibition of FLT1, FLT4, and PDGFRA, could give a biological substantiation to the complete remission as seen in the PHE patient (figure 4.5d).

To model PHE we overexpressed truncated FOSB in HUVECs. As the cell of origin is still enigmatic, we chose primary endothelial cells as a model because these cells are genetically normal (in contrast to cancer cell lines and transformed cells such as 293T). Histologically PHE does not form vessels making it difficult to formally prove endothelial cells are the precursor cells. We think however, given the fact that the tumor cells are known to express CD31, ERG, and FLI1, that endothelial cells, or their precursors, are the most likely precursors for these tumor cells (1, 2). Other candidates would have been

mesenchymal stem cells (MSC), which cannot be completely excluded as potential precursors for PHE. To overexpress truncated FOSB we used a lentiviral system. Truncated FOSB was under control of a constitutively active CMV promoter. We showed that truncated FOSB is able to regulate the *SERPINE1* promoter and that *FLT1* and *PDGFRA* are upregulated. We confirmed that the pattern of expression of these downstream target genes showed a similar trend in the patient's tumor tissue, both using qPCR (for *FAS*, *JAG1*, *HEY1*, *VWF*, *ADAMTS13*, *FLT1*, and *PDGFRA*) compared to normal HUVECs and using IHC (for KIT). However, a formal comparison with the patient tumor biopsy is difficult as the tumor biopsy also contains 40% normal cells and there was no normal tissue from the patient available. As truncated FOSB is constitutively active due to the CMV promotor, our model is not suited to compare the effect of telatinib on proliferation in normal HUVECs with HUVECs overexpressing truncated FOSB. Given the fact however that patients tolerate a high dose of telatinib (comparable to the concentration we used in vitro) for an extended period of time (12), it is likely that truncated FOSB expressing cells are more sensitive to telatinib than normal endothelial cells. We cannot completely exclude that the complete response seen in our patient was due to a spontaneous remission of the disease irrespective of the telatinib treatment. A literature search revealed one described case with a spontaneous remission of PHE as seen with PET/CT (18). Given the fact that the tumor progression halted shortly after starting telatinib treatment and that we showed in our in vitro model that telatinib could specifically interfere with the expression of its driver *SERPINE1-FOSB*, we think it is very likely that the remission observed here can be attributed to the telatinib treatment.

Based on our proposed mechanism of action for telatinib in PHE it is tempting to speculate that telatinib could also function as a targeted therapy in other vascular tumors. In atypical epithelioid hemangioma, a *ZFP36-FOSB* fusion is the most likely driving event (10). In this fusion *ZFP36* exon 1 fuses with exon 2 of *FOSB*. This fusion would result in a truncated FOSB protein driven by the *ZFP36* promoter, while retaining a slightly larger part of FOSB compared to the *SERPINE1-FOSB* fusion. Moreover, according to the DECODE database there are 14 AP-1 consensus binding sites in the *ZFP36* promoter making it most probable that the signaling cascade from the VEGF and PDGF receptors and self-regulation are important in driving expression of this fusion product. In both cases inhibition of the VEGF and PDGF receptors would likely lead to a reduction in expression of the fusion products as in both cases the fusion genes are downstream in the signaling cascade.

Three case reports described the mTOR inhibitors sirolimus, everolimus, and rapamycin as efficient treatment options for PHE (19–21). Reportedly, the AKT/mTOR signaling is, among other surface receptors, also downstream of VEGF-receptor signal-

ing (22). Moreover, it is also reported that AKT/mTOR signaling can lead to upregulation of AP-1 family members (23). Therefore, it is possible that treatment with the mTOR inhibitors would also result in a reduction in expression of the SERPINE-FOSB fusion protein leading to a similar inhibitory effect as telatinib treatment.

Based on our results, it is likely that other tyrosine kinase inhibitors could have a similar effect as telatinib in the treatment of PHE. Pazopanib is one of these tyrosine kinase inhibitors that target VEGF-receptors 1 to 3 and the PDGF-receptors, therefore showing a large overlap with telatinib. Pazopanib was approved as a treatment for soft tissue sarcomas, including angiosarcoma and epithelioid hemangioendothelioma, where standard therapy has failed (24).

In conclusion, we show that truncated FOSB protein, resulting from *SERPINE1-FOSB* fusion, is downstream of VEGF- and PDGF- receptor signaling, and that inhibition of signaling from these surface receptors reduces expression of the fusion protein. Moreover, we showed that *SERPINE1-FOSB* is capable of regulating its own promoter. Consequently, an initial reduction in expression of the *SERPINE1-FOSB* fusion would result in a further reduction as self-regulation is diminished.

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