FKBPL Is a Critical Antiangiogenic Regulator of Developmental and Pathological Angiogenesis

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Objective—The antitumor effects of FK506-binding protein like (FKBPL) and its extracellular role in angiogenesis are well characterized; however, its role in physiological/developmental angiogenesis and the effect of FKBPL ablation has not been evaluated. This is important as effects of some angiogenic proteins are dosage dependent. Here we evaluate the regulation of FKBPL secretion under angiogenic stimuli, as well as the effect of FKBPL ablation in angiogenesis using mouse and zebrafish models.

Approach and Results—FKBPL is secreted maximally by human microvascular endothelial cells and fibroblasts, and this was specifically downregulated by proangiogenic hypoxic signals, but not by the angiogenic cytokines, VEGF or IL8. FKBPL’s critical role in angiogenesis was supported by our inability to generate an Fkbpl knockout mouse, with embryonic lethality occurring before E8.5. However, whilst Fkbpl heterozygotic embryos showed some vasculature irregularities, the mice developed normally. In murine angiogenesis models, including the ex vivo aortic ring assay, in vivo sponge assay, and tumor growth assay, Fkbpl+/− mice exhibited increased sprouting, enhanced vessel recruitment, and faster tumor growth, respectively, supporting the antiangiogenic function of FKBPL. In zebrafish, knockdown of zFkbpl using morpholinos disrupted the vasculature, and the phenotype was rescued with hFKBPL. Interestingly, this vessel disruption was ineffective when zcd44 was knocked-down, supporting the dependency of zFkbpl on zCd44 in zebrafish.

Conclusions—FKBPL is an important regulator of angiogenesis, having an essential role in murine and zebrafish blood vessel development. Mouse models of angiogenesis demonstrated a proangiogenic phenotype in Fkbpl heterozygotes. (Arterioscler Thromb Vasc Biol. 2015;35:845-854. DOI: 10.1161/ATVBAHA.114.304539.)

Key Words: angiogenesis ■ CD44 ■ FKBPL ■ knockout ■ vasculature

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he vascular system fulfills the oxygen and nutrient requirements of tissues and organs, whilst maintaining tissue growth and repair. Vasculogenesis is a highly orchestrated process in embryonic development where de novo vessel formation takes place from angioblasts, the endothelial precursor cells.1 Subsequent sprouting from existing vessels, that is angiogenesis, leads to a vascular network of arteries, veins, and capillaries. In adult organisms, this process is quiescent; however, endothelial cells retain their capacity to differentiate into new vessels in response to angiogenic signals when required. Angiogenesis is deregulated in disease states; it is inadequate in diseases, such as stroke, ischemia, and diabetic wound healing;2 and excessive in cancer, inflammatory disorders, and eye diseases;3,4 thus, offering tremendous scope for therapeutic intervention.4 Many pro- and antiangiogenic strategies have been developed; although the proangiogenic approaches have not been successful to date, antiangiogenic therapies have been moderately effective in the treatment of cancer and eye diseases.5 Clinically approved antiangiogenic anticancer agents primarily target the VEGF pathway and have many limitations, such as increased resistance, toxicity, secondary metastases,7 and limited efficacy in certain cancer types, highlighting the need for novel agents targeting alternative pathways.

FKBPL-binding protein like (FKBPL), a novel member of the immunophilin protein family,5,9 is a potent secreted antiangiogenic protein targeting the CD44 pathway.12 FKBPL's critical role in angiogenesis was supported by our inability to generate an Fkbpl knockout mouse, with embryonic lethality occurring before E8.5. However, whilst Fkbpl heterozygotic embryos showed some vasculature irregularities, the mice developed normally. In murine angiogenesis models, including the ex vivo aortic ring assay, in vivo sponge assay, and tumor growth assay, Fkbpl+/− mice exhibited increased sprouting, enhanced vessel recruitment, and faster tumor growth, respectively, supporting the antiangiogenic function of FKBPL. In zebrafish, knockdown of zFkbpl using morpholinos disrupted the vasculature, and the phenotype was rescued with hFKBPL. Interestingly, this vessel disruption was ineffective when zcd44 was knocked-down, supporting the dependency of zFkbpl on zCd44 in zebrafish.

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FKBPL is also a prognostic biomarker for breast cancer and predictive for endocrine therapy. Therapeutic peptide derivatives of FKBPL, AD-01 and ALM201, have highly potent antiangiogenic activity, and because of their CD44-mediated effect, these agents are also able to target CD44-positive cancer stem cells, further strengthening their therapeutic potential. Following extensive preclinical validation, ALM201 has now completed toxicological evaluation in preparation for phase I clinical trials in cancer patients. Although the robust antiangiogenic effects of FKBPL and its therapeutics have been well characterized, the role of endogenous FKBPL in physiological and developmental angiogenesis is not. Here we address these questions using cell lines, knockout (KO) mice, and zebrafish models. We have developed an Fkbpl heterozygote mouse model and characterized the functional role of FKBPL in a range of in vivo and ex vivo angiogenesis end points. Furthermore, we have used GFP-zebrafish that provides an easy and strong model for evaluating developmental angiogenesis. We report that the antiangiogenic protein, FKBPL, plays a critical role in embryonic development, and its secretion is regulated by hypoxia. Fkbpl heterozygous mice upregulated angiogenesis in all models evaluated, strengthening the biological role of FKBPL in angiogenesis and supports FKBPL-based diagnostic and therapeutic interventions as they advance to clinical trials.

Materials and Methods
Materials and methods are available in the online-only Data Supplement.

Results
FKBPL Secretion Is Regulated by Hypoxia
We have previously reported that extracellular FKBPL exerts its antimigratory effects via CD44, and this effect can be reversed using a blocking antibody specific to the active angiogenic site of

Figure 1. Regulation of FK506-binding protein like (FKBPL) secretion. A, ELISA demonstrating that FKBPL was maximally secreted by endothelial (HMEC-1) and fibroblast (AGO-1522) cell lines, in comparison to tumor cell lines, and B, its secretion was specifically downregulated under hypoxia in HMEC-1 cells; intracellular protein (C) or mRNA (D) levels assessed by qRT-PCR did not change. Pro-angiogenic cytokines, VEGF, 25 ng/mL, or IL8, 1 nmol/L, did not alter FKBPL secretion (E) or intracellular (F) levels. A log phase 24 h old monolayer was subjected to different oxygen tensions or cytokine treatments for 24 h and the spent medium; RNA and protein from cell lysates were harvested. FKBPL levels were quantified by ELISA of 50-fold concentrated spent medium and normalized to cell number at the time of harvest. Data points are means±SEM. n≥3. **P<0.01 paired t test.
FKBPL. When secreted levels of FKBPL from various cell lines were evaluated by ELISA, maximal secretion (15–20 ng/10^7 cells and ≈7 ng/10^7 cells) was observed in endothelial cells, human microvascular endothelial cells (HMEC-1), and normal human fibroblasts, AGO-1552, respectively. Cancer cell lines or the normal breast epithelial cell line, MCF10A, secreted lower levels, ≤1.5 ng/10^7 cells (Figure 1A). The secretion from HMEC-1 was specifically inhibited when cells were cultured in a proangiogenic hypoxic environment (0.1% O_2) for 24 h (Figure 1B), whereas FKBPL mRNA or intracellular FKBPL protein levels remained unchanged (Figure 1C and 1D). HIF-1 increased upon hypoxic stimulation, acting as a positive control. Other proangiogenic stimulators, such as VEGF, IL-8, and bFGF, did not significantly alter FKBPL secretion, protein or mRNA expression (Figure 1E and 1F; Figure 1 in the online-only Data Supplement). Having established that FKBPL secretion was inhibited by proangiogenic hypoxic signaling, we proceeded to evaluate its endogenous role in development and pathological angiogenesis using knockout models.

**FKbpl Heterozygous Knockout Mice Appear Normal but Homozygous Knockout Is Embryonically Lethal**

The Fkbpl-targeted allele was deleted in the exon 2 region (Figure IIAi and IIaII in the online-only Data Supplement) and following germline transmission resulting progeny were genotyped; the presence of one band at 744 bp corresponded to Fkbpl wildtype allele and an additional band at 526 bp corresponded to the Fkbpl-targeted allele (Figure 2A(i)). Intercrossing of Fkbpl<sup>++/+</sup> mice did not yield any Fkbpl<sup>+/-</sup> mice, with over 50 progeny, indicating that the homozygous KO of Fkbpl was embryonically lethal. The Fkbpl<sup>+/-</sup> mice retained the neomycin selective marker; therefore, to exclude any interference from expression of this marker, it was deleted using CRE<sup>td</sup>/mi mice with 2 sequential crosses with Fkbpl<sup>+/-</sup> mice to obtain Fkbpl<sup>−/−</sup> mice (Figure IIaIII in the online-only Data Supplement) that were genotyped to confirm the presence of 2 bands at 537 and 240 bp for Fkbpl<sup>−/−</sup> mice and a single band at 240 bp for identifying the Fkbpl<sup>−/−</sup> mice (Figure 2Aii). Subsequently, embryos from timed matings were analyzed at E8.5-E13. The presence of empty yolk sacs and resorbed embryos was observed at E8.5 and E9.5 (Figure 2Aiii), suggesting lethality within the Fkbpl<sup>neo/neo</sup> line, confirmed by genotyping of the yolksacs (Figure 2Aiii); intact Fkbpl<sup>neo/neo</sup> embryos did not demonstrate any gross phenotypic changes. Embryonic lethality was also confirmed in the Fkbpl<sup>−/−</sup> mice because of the inability to obtain Fkbpl<sup>−/−</sup> mice with several (>50) intercrosses of Fkbpl<sup>−/−</sup> mice.

**Figure 2. Fkbpl knockout mice and embryo characterization.** A. Genomic PCR of (i) Fkbpl<sup>++/+</sup> (lanes 1–4) heterozygotes and Fkbpl<sup>−/−</sup> littermate controls (lane 5) and (ii) Fkbpl<sup>−/−</sup> (lanes 1–3) and Fkbpl<sup>++/+</sup> (lane 4) genotypes. (iii) Timed matings of the Fkbpl heterozygous crosses, demonstrating E9.5 embryos showing empty or resorbed yolk sac (arrows) and genotyping of the embryonic yolk sac; Fkbpl<sup>++/+</sup> (lane 1.8), Fkbpl<sup>−/−</sup>neo/neo (lanes 1.1–1.4, 1.9) and Fkbpl<sup>++/+</sup>neo/neo (lanes 1.5–1.7; boxed) alleles. Homozygous Fkbpl<sup>neo/neo</sup> knockout corresponded to resorbed embryos. B. FK506-binding protein like (FKBPL) expression in adult mice (i) real-time quantitative PCR analysis of RNA from tissue lysates of murine organs, validating the effect of genomic knockdown on Fkbpl mRNA and (ii) ELISA of serum for secreted FKBPL. Reduced levels in Fkbpl<sup>−/−</sup> and Fkbpl<sup>−/−</sup> mice in comparison to their Fkbpl<sup>++/+</sup> littermates. Murine organs were harvested in RNAlater, followed by RNA extraction qPCR quantification. Serum was collected from age-matched mice bleeds. Data points are mean±SEM. *P<0.05, **P<0.01, ***P<0.001 (B(i) unpaired t-tests and B(ii) 1-way ANOVA, Tukey multiple comparison). C. Expression of FKBPL and endomucin in intersomitic vessels of Fkbpl<sup>−/−</sup> and Fkbpl<sup>−/−</sup> mice at E11.5. Transverse sections at the level of an intersomitic vessel showing merged images of FKBPL expression in Fkbpl<sup>−/−</sup> embryo (a, 40×; b, 63×, magnification), single channel (ai, bi) FKBPL (green) and (aII, bII) endomucin (red). FKBPL is strongly expressed in endomucin<sup>++/+</sup> cells (yellow merge; small arrows) and in nucleated rounded cells, in the lumen of blood vessels (big arrows). Intersomitic vessels show stereotypical pattern. Merged images (c, d) showing reduced FKBPL expression in a cluster of round nucleated cells located within the lumen of an endomucin<sup>++/+</sup> intersomitic blood vessel in Fkbpl<sup>−/−</sup> mice. Intersomitic vessels show normal pattern but seem locally distended by cluster of intraluminal cells (big arrow). Single channel images: (c) FKBPL (green) and (cII) endomucin (red). All slides were counterstained with DAPI (blue). Scale bar=50 μm in a and c, 40× and 10 μm in b and d, 63×.
**Fkbpl**KO mice were then further characterized. Genomic KO of one Fkbpl allele resulted in ≈2-fold reduction of Fkbpl mRNA in all organs analyzed (Figure 2Bi). Reduced levels of Fkbpl expression because of loss of one allele was also confirmed in the FkbplKO mice (Figure IIC in the online-only Data Supplement). However, serum FKBPL levels were downregulated up to 4-fold in the FkbplKO or FkbplKO mice in comparison to FkbplKO littermates (Figure 2Bii); there was no significant difference in FKBPL levels between FkbplKO or FkbplKO mice. The FkbplKO and FkbplKO mice developed normally with no overt abnormalities, and the organ histology appeared normal (Figure IIB in the online-only Data Supplement).

**Embryonic Expression of FKBPL and Effects on Vasculature**

During early development (E11.5), FKBPL was strongly expressed in endothelial (endomucin+) cells of the blood vessels, as shown by yellow (red–green merged) staining (Figure 2Ca and 2Cb, small arrows), consistent with Figure 1A. FKBPL was also expressed in nucleated rounded cells located within blood vessels (Figure 2Ca and 2Cb, big arrows; video in the online-only Data Supplement). Additionally, FKBPL was expressed in many other cell types, as an intense subcellular spot, closely associated with the nucleus (Figure 2Ca and 2Cb, small arrows). In accordance with the qPCR data presented in Figure 2Bi, heterozygote mice showed reduced FKBPL immunostaining (Figure 2Cc and 2Cd). Interestingly, accumulation of rounded FKBPL+ cells was often seen within the blood vessels of FkbplKO embryos (Figure 2Cc and 2Cd, big arrow). However, this did not affect the overall development of the FkbplKO mice because the newborn and adult mice appeared normal.

**Increased Ex Vivo Sprouting of Aortae in Fkbpl Heterozygotic Mice**

Further characterization of Fkbpl KO in angiogenesis was performed by assessing the effect on aortic sprouting ex vivo. The aortae from FkbplKO mice showed enhanced sprouting and disordered branching (Figure 3A). Quantification revealed a statistically significant increase (≈2 fold) in vessel branching and vessel length (Figure 3B). The endothelial origin of the sprouts was confirmed by staining aortic rings with endomucin (Figure IV in the online-only Data Supplement). The FkbplKO littermates showed an identical phenotype in the aortic sprouting to that of WT C57Bl6 mice as expected (Figure IIIA in the online-only Data Supplement). FkbplKO aortae showed an

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**Figure 3.** FK506-binding protein like (FKBPL) regulates ex vivo sprouting of vessels from mouse aorta. Representative photomicrographs (A) and vessel measurements (B) of aortic rings showing enhanced sprouting of vessels from the FkbplKO aortae, C, Inhibition of sprouting mediated by AD-01 (100 nmol/L). D, Additive effect of VEGF (30 ng/mL) treatment on vessel sprouting in FkbplKO and FkbplKO aortae. Sliced, excised aortae were embedded into matrigel±AD-01 or VEGF and vessel sprouting evaluated microscopically after 10 days. Data points are mean±SEM. n≥3. *P<0.05, **P<0.01, ***P<0.001 (1-way ANOVA, C, D; Tukey multiple comparison post tests).
enhanced pattern of sprouting identical to Fkbpl<sup>+/neo</sup> aortae (Figure IIIB in the online-only Data Supplement), indicating that the 2 murine Fkbpl<sup>+/neo</sup> and Fkbpl<sup>−/−</sup> strains have a similar phenotype. To demonstrate that this was caused by a reduction in the levels of the antiangiogenic protein, FKBPL, AD-01, the therapeutic peptide derived from the antiangiogenic domain of FKBPL, was added to the assay conditions. As expected, this abrogated the enhanced sprouting observed in Fkbpl<sup>+/neo</sup> aortae because of restoration of the FKBPL antiangiogenic function by complementing the activity levels. The angiogenic activity of the Fkbpl<sup>+/+</sup> aortae was also significantly inhibited as expected (Figure 3C). Treatment of the aortic rings with VEGF enhanced the sprouting of Fkbpl<sup>+/−</sup> aortae and had an additive enhancement effect on Fkbpl<sup>+/neo</sup> aortae (Figure 3D), suggesting that FKBPL-mediated sprouting is independent of VEGF angiogenesis pathway.

**Fkbpl Heterozygous Mice Support Enhanced Vessel Recruitment In Vivo in the Sponge Assay**

The effect of Fkbpl ablation was further assessed in vivo in a sponge assay. Polyether sponges were implanted into Fkbpl<sup>+/neo</sup>, Fkbpl<sup>+/−</sup>, and Fkbpl<sup>+/+</sup> mice, and microvessel recruitment was stimulated with bFGF. H&E stained sections of sponges from Fkbpl<sup>+/neo</sup> mice had a significantly higher number of small (40%) and big (50%) vessels (Figures 4A and 4B). The endothelial specificity of this effect was further confirmed using endomucin staining; a significantly higher number (15% to 20%) of endomucin-stained vessels were observed in sponges from Fkbpl<sup>+/neo</sup> (<P<0.05) and Fkbpl<sup>−/−</sup> (<P<0.005) in comparison to the Fkbpl<sup>+/+</sup> littermates. This enhanced angiogenesis, however, also corresponded with an increase in the number of erythrocytes leaking into the surrounding tissue (Figures 4E and 4F), indicating that vessels within FKBPL-deficient mice may be less robust.

**Higher Tumor Growth Rate and Tumor Angiogenesis in Fkbpl Heterozygous Mice**

Role of Fkbpl ablation in pathological angiogenesis was evaluated in the syngeneic Lewis lung carcinoma tumor model. Lewis lung carcinoma cells grown on the rear dorsum of Fkbpl<sup>+/neo</sup> or Fkbpl<sup>+/−</sup> mice had an increased growth rate in comparison to the Fkbpl<sup>+/+</sup> littermates (Figure 5A), corresponding to a significantly shorter survival, defined as time to reach >600 mm<sup>3</sup> in tumor volume (Figure 5B) in the Fkbpl<sup>−/−</sup> littermates.
heterozygotes. Immunohistology of the tumors for CD31 expression was performed to assess the effect on tumor vasculature; an increase in CD31-stained blood vessels (Figure 5C) was observed in Fkbpl+/neo and Fkbpl+/− mice. Gross examination of the blood vessels suggested an irregular structure, with the endothelium appearing thicker and less organized in tumors grown in Fkbpl+/neo and Fkbpl+/− mice in comparison to a well-defined and ordered vasculature in Fkbpl+/+ littermates (Figure 5D): a feature of enhanced angiogenesis.

Fkbpl Regulates Developmental Angiogenesis in the Zebrafish

Having established that KO of one allele of Fkbpl produced a proangiogenic phenotype in a range of murine assays, we proceeded to evaluate this effect in zebrafish. NCBI Standard Protein Blast analysis identified Similar to W AF-1/CIP1 stabilizing protein 39 isoform 2 (GenBank ID: XP_001923918.1) as the zebrafish orthologue to the human FKBPL protein (GenBank ID: NP_071393.2). ClustalW2 alignment showed 27% identity between the 2 sequences (Figure VA in the online-only Data Supplement). Analysis of the protein domains using SMART (Simple Modular Architecture Research Tool) showed a similar structure in the C-terminal tetra-trico peptide repeat (TPR) domains, typical of almost all FKBP family members (Figure VB in the online-only Data Supplement).

Microinjection of AD-01 into zebrafish embryos at 72 hpf inhibited the intersegmental vessels (Figure 6A), whereas scrambled AD-01 did not affect the vasculature and showed a similar pattern to uninjected embryos. Vessel abnormalities were seen in ≈10% embryos. However, this effect was statistically significant (P<0.05); 400 embryos were assessed to verify the effect. This demonstrated the effectiveness of the FKBPL antiangiogenic activity across species.

We also investigated the function of zfkbpl during zebrafish embryonic development by microinjection of zfkbpl-targeted morpholinos (MO). Injection of splice and translation blocking MOs (UTR MO), leading to formation of a nonfunctional Fkbpl protein impaired the formation of the dorsal longitudinal anastomotic vessels and intersegmental vessel in ≈85% of the injected embryos at 48 hpf (Figure 6B and 6C; P=0.006 for
UTR MO and P=0.01 for splice MO). Interestingly, this also reduced the number of vessels that form the caudal vein, making it look thinner. This vessel inhibition with zkfbpl knockdown is contrary to the earlier findings of enhanced angiogenesis with loss of one allele in our mouse model and could possibly be as a result of levels of Fkbpl. Conditional Fkbpl−/− KO models could help further identify any possible dosage-related effects that FKBPL might have. Notably, the UTR control MO and splice control MO did not alter the vasculature significantly (Figure 6B and 6C). Although the splice MO was clearly effective in the inhibition of zebrafish vasculature, the window of effective concentration was, however, close to that of the off-target curling of the tail. We therefore show the clearer UTR MO data images in Figure 6D. The major trunk and tail vessels (DA and CV) were not disrupted. Co-injection of hFKBPL mRNA with the UTR and splice MO rescued the phenotype in ≤91% of splice MO (P=0.002) and 83% of UTR MO–injected embryos (P=0.01; Figure 6B and 6C). The rescue of vascular disruption was also obtained with either full length N or C terminal hFKBPL mRNA. Interestingly, the vascular disrupting effects of zkfbpl UTR MO were not seen in the presence of a zcd44 MO (Figure 6C), supporting the dependency of Fkbpl on Cd44. This suggests that zFkbpl is also secreted, exerting its effect in a CD44-dependent outside-in signaling that we have previously reported for human cells. No effect of zcd44 MO was seen in any of the injected embryos, and CD44 UTR MO control served as negative control. The vasculature did not seem altered in 24-hpf injected embryos.

**Discussion**

FKBPL is emerging as an important anticancer protein having intra- and extracellular roles in tumor and endothelial
cells. Although FKBPL mediates its intracellular roles predominantly via its interaction with other proteins, facilitated by its conserved TPR domain, its antiangiogenic effects are mediated through secretion and inside out signaling via the CD44 pathway. FKBPL is a nonconventional secreted protein lacking the signal peptide domain associated with proteins secreted via the golgi-ER pathway. We have previously reported that the antiangiogenic activity of FKBPL is reversed in the presence of a blocking antibody targeted to its antiangiogenic site, highlighting the role of the secreted protein in migration and angiogenesis.

Here we observe that FKBPL is mainly secreted from endothelial and fibroblast cell lines (10- to 15-fold higher than cancer or epithelial cells), the 2 cell types with angiogenic and wound healing functions. Importantly, FKBPL secretion is downregulated under hypoxia, a principal angiogenic stimulus that upregulates a range of angiogenic cytokines. This hypoxia-mediated regulation of FKBPL occurs specifically at the secreted level as the intracellular protein and RNA levels remain unaffected, further supporting the role of extracellular FKBPL in the regulation of angiogenesis. Interestingly, although VEGF is the proximate central angiogenic factor to hypoxia, regulating other downstream angiogenic events factors, it did not have any effect on FKBPL’s intra- or extracellular levels. Other angiogenic cytokines, IL-8 and FGF, did not affect FKBPL levels either. This further strengthens our hypothesis that FKBPL is upstream or independent of these pathways.

Although the role of FKBPL on tumor angiogenesis has been extensively investigated; its endogenous role in physiological or developmental angiogenesis has not yet been elucidated. Here we interrogated this function by developing an Fkbpl KO mouse model. The Fkbpl allele was targeted at the exon 2 region, leading to the complete deletion of the Fkbpl open reading frame (ORF), resulting in successful generation of mice heterozygous for Fkbpl. Attempts at generation of homozygous Fkbpl mice using Fkbplneo or Fkbpl−/− crosses were unsuccessful, suggesting that the homozygous Fkbpl KO is embryonically lethal, potentially because of its important and essential role in murine development. Other angiogenic regulators demonstrating embryonic lethality are VEGF-A, DLL4, the Notch pathway genes, and angiopoietin, whereas bFGF-null mice do not develop vascular defects, and inactivation of PlGF or VEGF-B genes does not result in any major development abnormalities. Nevertheless, modest overexpression of VEGF results in excessive angiogenesis in mice, leading to severe abnormalities and embryonic lethality. Using timed matings, we identified the Fkbpl KO–mediated embryonic lethality occurred at ≤8.5 days. This corresponds with early vasculogenesis in the mouse embryo, which commences around E7.0 when angioblasts entering the embryo aggregate to form major vessels.

Further analysis of both Fkbpl heterozygous strains for expression of FKBPL mRNA and secreted levels correlated with the genotype and confirmed the absence of dosage compensation in these mice. We therefore proceeded to characterize the embryonic development of Fkbplneo mice. In accordance with the qPCR results, confocal microscopy images of intersomatic blood vessels (at comparable level of the body axis) indicate that E11.5 Fkbpl−/neo mice showed significantly reduced levels of FKBPL compared with E11.5 Fkbpl+/+. FKBPL was strongly expressed in cells present in the lumen of blood vessels resembling primitive nucleated erythroblasts. This would seem to be consistent with its function as an angiogenesis regulator. The fenestrated morphology of vessels with clustering of FKBPL+ cells in Fkbpl−/neo embryo sections possibly indicate finer, easily distorted vessels, poor circulation, or a decreased number of FKBPL+ cells. The distinct morphology of the endothelial cells in Fkbpl−/neo embryos is consistent with the FKBPL-mediated effects on the cytoskeleton and the actin-tubulin dynamics as previously observed. These abnormalities in the embryonic vasculature, however, did not affect subsequent development, and no abnormality was observed in the histology of the organs. However, possible effects on longevity and age-related abnormalities remain to be investigated. To further evaluate the role of FKBPL in angiogenesis, induced models of angiogenesis were used. Ex vivo, aortae excised from Fkbpl−/− mice demonstrated ordered sprouting; however, this process appeared to be significantly enhanced in Fkbpl−/neo, and vessel sprouts were highly branched and numerous. The increase in sprouting in FKBPL-deficient vessels is most likely because of its effect on endothelial cell migration rather than enhanced endothelial cell proliferation; our previous studies clearly demonstrate that FKBPL or its peptide derivatives do not affect endothelial cell viability. The reversal of this enhanced sprouting obtained in Fkbpl−/neo aortae treated with AD-01 supported the specificity of its antiangiogenic activity. Furthermore, the additive effect of Fkbpl−/neo vessel sprouting obtained with VEGF treatment supports the hypothesis that FKBPL-mediated effects are independent of the VEGF pathway.

In an in vivo sponge assay, the increased vessel recruitment observed in Fkbpl−/neo and Fkbpl−/− mice confirmed their proangiogenic phenotype in vivo. The heterozygote vessels in the sponges appeared leaky on visual quantification. Irregular and increased vessel number was also observed in the Lewis lung carcinoma tumors grown in Fkbpl−/neo or Fkbpl−/− mice. Clearly, stromal FKBPL appeared to play a regulatory role in tumor growth and vascular development in this model; tumors in Fkbpl−/− heterozygote mice grew faster with an increase in tumor angiogenesis. The role of tumor microenvironment in the regulation of tumor growth is indeed critical and regulates key processes of angiogenesis, metastasis, and cancer stem cell differentiation and is one of the hallmarks of tumorigenesis.

To understand the mechanism of angiogenesis and its role in development, we used the zebrafish (Danio rerio) model. The FKBPL homologue (30% similarity) in zebrafish showed a similar structure in the C-terminal TPR domains, characteristic of the immunophilin family. The inhibition of intersegmental vascular formation in the zebrafish embryos with AD-01 treatment correlates to its well-characterized antiangiogenic effects observed earlier, as well as in this study. The endogenous role of Fkbpl in the fish model evaluated using morpholinos targeting the zfkbpl splice junction and the UTR resulted in an impairment of dorsal longitudinal anastomotic vessels and intersegmental vessel formation rescued with coinjection of either full length or N or C
terminal hFKBPL mRNA. This indicated the conservation of FKBPL function across species. Interestingly, this function was dependent on zCd44 as the zfkbp1 MO-mediated vessel impairment was abrogated in the zcd44 morphant zebrafish embryos. This confirms our earlier studies on the CD44-dependent effect of FKBPL on migration of tumor and endothelial cells, further strengthening the role of zCd44 as zFkbpl’s extracellular membrane target. The disruption in the vessels suggests that complete loss of zFkbpl inhibits vessel formation or perhaps formation of nonrobust vessels because of loss of its antiangiogenic activity. Indeed, tight regulation of VEGF is essential for a functional vasculature and development; zebrafish studies with VEGF overexpression, as well as zvegf morphant embryos, have reported defective vasculature, resulting in pericardial edema and abnormal blood cell accumulation. Alternatively, the complete loss of FKBPL, as well as excess FKBPL (overexpression and exogenous addition), may have inhibitory effects on angiogenesis. Dosage-dependent effects have been observed for other regulators of angiogenesis. For example, loss of one allele of DLL4 results in excessive and nonproductive angiogenesis, whereas Dil4 overexpressing and Dil4 KO mice both show impaired wound healing and antiangiogenic effects. Similar effects have been reported with focal adhesion kinase-deficient mice, where loss of one allele promoted angiogenesis and loss of both alleles inhibited it. Similarly, RGD (arginine-glycyl-aspartic acid) mimetics that target integrins show differential dosage-dependent effects on angiogenesis. Further studies using conditional Fkbpl−/− mice to allow both spatial and temporal ablation of FKBPL expression will be required to further elucidate the effect of FKBPL dosage.

Although the data presented here strongly suggest a role for FKBPL as a potent antiangiogenic mediator, our previous data also supports a role for FKBPL as an intracellular regulator of estrogen receptor signaling through its association with Hsp90. Therefore, the FKBPL-mediated effects on the embryonic lethality, the differences in our mouse and zebrafish data and perhaps why tumor growth rates in our FKBPL-deficient mice are higher, might also suggest a role for FKBPL-mediated regulation of estrogen receptor signaling. Indeed, the regulation of angiogenesis by estrogen and signaling via the Notch pathway has been previously reported. However, this is beyond the scope of the current article and would need to be further evaluated in future studies.

In conclusion, we have identified an essential role for FKBPL in murine embryonic and zebrafish vascular development. Loss of one Fkbpl allele in mice gives rise to a strong proangiogenic phenotype. Thus, deregulation of FKBPL levels could have serious implications for diseases associated with aberrant angiogenesis, widening the scope for the FKBPL-based clinical candidate, ALM201.

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Disclosures

None.

References

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therefore provides further support in enhancing the therapeutic and biomarker potential of FKBPL.

FKBPL also has potential as a biomarker of angiogenesis and its secreted levels, offering ease of detection. This study supports a possible role in diseases other than cancer, such as cardiovascular and wound healing disease, thus extending the therapeutic utility of FKBPL.

FKBPL levels can therefore be exploited for therapeutic purposes. Reduced FKBPL levels, leading to enhanced angiogenesis, implicates the role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. Mech Dev 2001;108:29–43.


Significance

Secreted FK506-binding protein like (FKBPL) and its peptide derivatives inhibit cell migration and tumor angiogenesis by targeting CD44. We report that secretion of FKBPL is downregulated under the proangiogenic stimulus of hypoxia, independent of VEGF pathway. Using knockout mice and zebrafish models, we have established that FKBPL is essential for early embryonic development. Moreover, it is a critical regulator of angiogenic process; loss of one Fkbpl allele results in enhanced angiogenesis in experimental and tumor disease models. Modulation of FKBPL levels can therefore be exploited for therapeutic purposes. Reduced FKBPL levels, leading to enhanced angiogenesis, implicates a possible role in diseases other than cancer, such as cardiovascular and wound healing disease, thus extending the therapeutic utility of targeting FKBPL. FKBPL also has potential as a biomarker of angiogenesis and its secreted levels, offering ease of detection. This study therefore provides further support in enhancing the therapeutic and biomarker potential of FKBPL.
FKBPL Is a Critical Antiangiogenic Regulator of Developmental and Pathological Angiogenesis
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MATERIALS AND METHODS

Cell lines and cultivation: Human microvascular endothelial cells (HMEC-1) cells were obtained from the Centre of Disease Control, (Atlanta, USA); human fibroblast cell line, AGO-1552 from Coriell Institute for Medical Research (Camden, NJ), non-tumour human breast cells, MCF10A, PC3, ZR-75, L132, MCF7 and MDA-MB-231 from the American Type Culture Collection (ATCC, LGC, UK). All cell lines were authenticated by short tandem repeat (STR) profiling carried out by the suppliers and routinely tested for Mycoplasma. All cell lines were cultivated at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}/95% O\textsubscript{2} in appropriate medium supplemented with 10% foetal calf serum (FCS) (PAA, UK); HMEC-1 cells in MCDB-131. (Invitrogen, UK), supplemented with epidermal growth factor (EGF, 10 ng/ml) (Roche, UK) and L-glutamine (10 mM) (Invitrogen, UK), MDA-MB-231, MCF7, ZR75 cells in DMEM (Invitrogen, UK), PC3 cells in RPMI 1640 (Invitrogen, UK) MCF10A in DMEM F12 (Invitrogen, UK). 5 % horse serum, 5 ml pen strep, 20 ng/ml EGF, 10 µg/ml hydrocortisone, 100 ng/ml cholera toxin and AGO-1552 in Eagle’s minimum essential medium supplemented with non-essential amino acids (Invitrogen, UK) and 20% Foetal bovine serum.

Transgenic mice: C57BL/6N mice were used for microinjection of the Fkbpl-targeted embryonic stem cell line, JM8A3. The vector, ES cell(s), and/or mouse strain used for this research project was generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). NIH grants to VelociGene at Regeneron Inc. (U01HG004085) and the CSD Consortium (U01HG004080) funded the generation of gene-targeted ES cells for 8500 genes in the KOMP Program and archived and distributed by the KOMP Repository at UC Davis and CHORI (U42RR024244). The targeting vector and the Fkbpl-targeted (exon 2 deletion and insertion of a generic targeting cassette) ES cells were obtained from KOMP (USA); exon 2 of Fkbpl was deleted. (Fkbpl - IKMC Project: 41363; Clone number: EPD0466_1_C01), obtained from KOMP (UC Davis Repository, USA) were microinjected at MRC Harwell (UK) into C57BL/6N mice mouse blastocysts to establish germ-line transmission. The resulting chimeric offspring were bred with C57BL mice to obtain germline transmission of the mutated Fkbpl allele and obtain Fkbpl\textsuperscript{+/neo} mice. Subsequently, Fkbpl\textsuperscript{+/neo} mice were crossed with B6N-TgN (ACTB-Cre)3Mrt/H mice (βactin-Cre Tg) to ubiquitously remove the floxed neomycin selective marker from the Fkbpl mutated allele (Fkbpl\textsuperscript{+/}). Fkbpl\textsuperscript{+/} mice were further bred to remove the β-actin-Cre from the germline. The resulting Fkbpl\textsuperscript{+/−} and Fkbpl\textsuperscript{+/+} lines was used in comparison to their Fkbpl\textsuperscript{−/−} littermates for further analyses.

All animal experiments were approved by both the Department of Health Social Services and Public Safety Northern Ireland (DHSPPNI) and the University Animal Welfare Ethical Review Board under Project Licences PPL 2678 & PPL2660, carried out in accordance with the Animal (Scientific Procedures) Act 1986 and conformed to the current UKCCCR guidelines.

Genomic DNA extraction and PCR: Genotyping of mice was carried out using genomic DNA PCR. Genomic DNA from mouse ear snips or embryonic yolk sacs was prepared by dissociating the tissue in lysis buffer (Tris, EDTA, NaCl, SDS, Proteinase K) at 55°C, 900 rpm, overnight, followed by DNA precipitation with isopropanol and 70% ethanol wash. Extracted DNA was suspended in water and used for PCR amplification of Fkbpl allele using specific primers. Forward primers, Fkbpl-002 designed upstream of the generic targeting cassette together with reverse primer Fkbpl-003 amplified within the exon 2 region present in the full length WT chromosome. Therefore, amplification with Fkbpl-002 and Fkbpl-003 primers produced a fragment of 703 bp, respectively, if the cells have a wild-type allele. The primer, Common-en2-R, is located within the generic targeting cassette, so if the targeting construct has inserted correctly, exon 2 (containing Fkbpl-003 binding region) will not be present on one allele, but the Common-en2-R binding region will be present, therefore the primers will amplify the region between Fkbpl-002 and Common-en2-R producing fragment of 526 bp. This will be seen on the gel at approximately 500 bp, indicating a homozygote, or two bands at 500 and 700 bp, indicating a heterozygote. Forward primer Fkbpl-004 (d, Fig 2A) and reverse primers Fkbpl-005 and Fkbpl-Cre (e, Fig. 2A) were used to differentiate the Fkbpl\textsuperscript{−/−} and Fkbpl\textsuperscript{+/−} genotypes.

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ELISA: Secreted FKBPL from cell lines and murine serum was quantitated using either human (SEL523Hu) or murine (SEL523Mu) ELISA kit (Uscn Life Science Inc., Wuhan). 2x10⁶ cells were seeded in p90 dish in 5 ml normal medium and cells were grown for 48h. For hypoxia experiment, cells were grown in normal cultivation conditions for 24 h and then subjected to either normal (21%) or 0.1% O₂ cultivation. Similarly VEGF (25ng/ml, cat no 100-20 (B), Peprotech) and IL8 (1 nM, Peprotech) treatments were applied for 24 h. 5 plates were used for each treatment conditions. The pooled spent medium from 5 plates was filtered and concentrated 50 fold using Amicon ultra centrifugal concentrators (5 kDa cut off, Merck Millipore, MA, USA) and subjected to ELISA using manufacturer’s instruction. On the day of harvest total cell count was estimated and secreted protein was normalised to the cell number obtained.

Murine serum was prepared from mouse blood harvested by cardiac puncture, coagulated for 10 min at room temperature (RT) and centrifuged at 10,000 rpm for 10 min. The supernatant was used for ELISA as described above. If necessary, samples were stored at -80°C until required.

Western blotting: Cell monolayers were grown as described above, lysed and subjected to immunoblotting with FKBPL, GAPDH or HIF-1 antibody as described earlier. Briefly, Cell lysates harvested using RIPA (Radio-Immuno Precipitation Assay) buffer supplemented with protease and phosphatase inhibitor cocktails (Roche, UK). Protein was quantified by BioRad reagent or nanodrop Spectrophotometer (2000c, Thermo Scientific) and 20 to 40 µg protein was loaded for each sample. Cell lysates were reduced in Laemmli buffer (Sigma, UK) and subjected to western blotting as reported earlier blots were probed with specific primary antibody and appropriate HRP-linked secondary IgG (GE Healthcare, UK) at 1:10000, followed by detection with Chemiluminescent Substrate (Millipore, UK).

Antibodies. FKBPL (Proteintech, USA Cat No: 10060-1-AP); HIF-1 (BD Biosciences, Cat No: 610958), endomucin (Santa Cruz Biotechnology, USA Cat No: sc-65495.), CD31 (Abcam PLC, Cambridge, UK Cat No: ab28364.), anti-smooth muscle actin (Abcam PLC Cambridge UK Cat No: ab7817), Alexa Fluor® 488 Goat Anti-Rabbit IgG (Life Technologies, UK Cat no A-11034), and Alexa Fluor® 568 Goat Anti-Rat IgG (Life Technologies, UK Cat no. A-11077) anti-GAPDH and Actin (Sigma, UK cat: G9545 and A4700 respectively), Omnimap Anti-Rb HRP (Ventana 760-4311) or anti-Rat HRP (Ventana 760-4457). All antibodies were used at 1:1000 for immunoblotting and at specified dilutions for immunohistochemistry, unless otherwise stated.

Real time Quantitative PCR and mRNA extraction:

MDA-MB-231 cells were seeded at 1 x 10⁶ cells per P90 plate with 6 mL complete media. HMECs were seeded 2 x 10⁶ per B-90 plate with 6 ml complete media. Plates were incubated for 24 h and then placed either in hypoxic (0.1% O₂, 37°C, 5% CO₂) or normoxic conditions (37 °C, 5% CO₂) for 4 h or 24 h, or exposed to 30 ng/mL VEGF or bFGF before cell lysates were harvested.
The media from P90 plates was removed, 1 ml RNA-STAT 60 Reagent (Ambbio, UK) was added per plate, cell monolayers were scraped from the plate surface, collected in 1.5 ml Eppendorf tube, and pipetted up and down repeatedly to ensure cell lysis. The lysates were incubated at room temperature for 5 min and stored at -80°C. The use of RNAase free tubes and tips were used to minimise RNA degradation.

To process samples, 150 µl chloroform was added to each sample, vortexed for 30 seconds, and incubated at room temperature for 2 min. Samples were centrifuged at 14000 rpm for 30 sec at 4°C and the upper aqueous layer removed to a fresh tube. 500 µl isopropanol was added and the samples stored at -20°C overnight to allow RNA precipitation. Samples were then centrifuged at 14000 rpm for 30 min at 4°C. The supernatant was discarded, 500 µl RNase-free 70% ethanol was added to the pellet and the sample was mixed then centrifuged at 14000 rpm for 15 min at 4°C. The supernatant was discarded, and the pellets rewash in ethanol as above, before being allowed to air dry on ice, and resuspended in 50 µl RNase-free water for 5 min at 65°C. RNA purity was confirmed using the NanoDrop Spectrophotometer (2000c, Thermo Scientific), where 260/280 should be 2.0 and 260/230 should be 2.0 – 2.2. RNA samples were stored at -80°C.

**mRNA extraction from mouse tissue:** Mouse organs from Fkbpl+/+ and Fkbpl+/- mice were excised and stored in RNA later solution (Life technologies, UK) at -80°C. Samples were thawed immediately before use, and 30 mg of lung, liver, and kidney, and 20 mg of spleen was added to lysis buffer from the GeneJET RNA Purification Kit (Thermo Scientific, UK) in GentleMACS M tubes (Miltenyi Biotec, UK) and tissues disrupted using the RNA program on the GentleMACS Tissue Dissociator (Miltenyi Biotec, UK). Dissociated tissue was then processed as per the manufacturer’s protocol in the GeneJET RNA Purification Kit (Thermo Scientific, UK).

cDNA was prepared from 1 µg of extracted RNA using the Roche first strand cDNA synthesis kit (Roche, UK) and following the manufacturer’s protocol. cDNA was prepared for qRT-PCR using the Roche Lightcycler Probes 480 Master kit (Roche, UK) and Roche Realtime Ready TaqMan gene expression mono hydrolysis Probes (Roche, UK) for human FKBPL and human GAPDH were used to amplify the previously obtained cDNA on the LightCycler 480 RTPCR machine (Roche, UK). cDNA was prepared from 1 µg of extracted RNA using the Roche first strand cDNA synthesis kit (Roche, UK) and following the manufacturer’s protocol. cDNA was prepared for qRT-PCR using the Roche Lightcycler Probes 480 Master kit (Roche, UK) and Roche Realtime Ready TaqMan gene expression mono hydrolysis Probes (Roche, UK) for human FKBPL and human GAPDH, and Applied Biosystems TaqMan Gene expression assays for murine Fkbpl (Mm00498192_s1) and Gapdh (Mm99999915_g1) (Applied Biosystems) were used to amplify the previously obtained cDNA on the LightCycler 480 RTPCR machine (Roche, UK).

The resulting crossing points (Cp - first cycle where a sample shows logarithmic amplification) were calculated using the Roche LightCycler 480 software, and quantified using the standard curve efficiency. Sample Cp values were corrected to GAPDH levels.

**Immunohistology:** Formalin-fixed, paraffin-embedded murine organs, tumours or sponges as appropriate were sectioned into 3 µm section using a Leica ASP200 automated tissue processor and Leica RM 2135 microtome and subsequently stained with haematoxylin and eosin (H and E) or anti-CD31/anti-endomucin antibodies. CD31 and endomucin staining was carried out on a Ventana Discovery XT, using “mild” (8 min at 95C plus 12 min at 100C) or ‘standard’ (8 min at 95C plus 28 min at 100C) cell conditioning (CC1 Ventana 950-500). Primary antibodies were applied at a dilution of 1/50 for 1 hour at room temperature. Detection was performed using Omnimap Anti-Rb HRP (Ventana 760-4311) or anti-Rat HRP (Ventana 760-4457) and Ventana Chromomap DAB kit (Ventana 760-159) as per Ventana Discovery XT protocol. Sections were subsequently lightly counterstained with Mayer’s haemalum to show nuclear detail. Slides were scanned using the Aperio Scanscope CSO 5200 scanner. Each specimen was scanned at bright field 40x magnification. The Aperio CSO digitizes entire slides at high resolution, using software applications to view and share digital images. It consists of a retractable stage and optical components, using a line scanning system.
to produce a high quality and high resolution scan. Further quantification of CD31 and endomucin stained vessels were made on the digital images.

**Embryo analysis:** Timed matings were set up with Fkbpl<sup>+/neo</sup> male and female mice and plug formation was monitored closely every day and embryos from E8.5, 9.5, and E11.5 were harvested for genotyping and further analysis. Genotyping was carried out using PCR on the embryonic yolk sac. For immunostaining, E11.5 Fkbpl<sup>+/neo</sup> and Fkbpl<sup>+/+</sup> Embryos were fixed in 4% Paraformaldehyde/PBS overnight at 4°C. Embryos were then removed, rinsed in PBS and soaked in Sucrose solution (Succharose 15%/Phosphate buffer 0.12 M) overnight at 4°C. Embryos were then soaked in gelatine solution (PBS/succharose 15%/gelatine 7.5%) at 37°C for 30 min and embedded in liquid gelatine in a P35 plate to allow orientation for transverse sectioning. These were allowed to cool before cutting into blocks and mounting on a cork base using OCT (VWR, UK). Blocks were frozen in isopropanol cooled to -65°C for 1 min, before storage at -70°C. Blocks were sectioned into 14 µm sections using a cryostat and put on SuperFrost™ Plus slides (Fisher Scientific, UK). Slides were washed with PBS at 37°C for 15 min to remove gelatine, before two 5 min washes with PBS at room temperature and permeabilisation with 0.1% PBS-Triton for 30 min at room temperature. Slides were blocked for 30 min with blocking solution/10% serum/1% triton. FKBPL primary polyclonal rabbit antibody (Proteintech, UK) and Endomucin primary monoclonal rat antibody (Santa Cruz Biotechnology, USA) were added to the slides, both diluted 1:100 in PBS, and covered with a coverslip, overnight at 4°C. Slides were given five 5 min washes in PBS and the relevant secondary antibodies (Alexa Fluor® 488 Goat Anti-Rabbit IgG (Life Technologies, UK), and Alexa Fluor® 568 Goat Anti-Rat IgG (Life Technologies, UK)), diluted 1:500 in PBS were added for 2 h at room temperature. Slides were given five 5 min washes in PBS and on the final wash, 10 µL of DAPI (Life Technologies, UK) was added, before having excess moisture removed. Two drops of VECTASHIELD HardSet mounting medium with DAPI (Vector Labs, UK) was added, and a coverslip placed on top. Slides were stored at 4°C for imaging.

**Confocal microscopy:** Slides were imaged on a Zeiss LSM 710 at 40x and 63X, magnification with images taken at the same transversal section of the neural tube. Images were processed using Fiji imaging software where channel 1 (blue) showed DAPI, channel 2 (green) showed FKBPL, and Channel 3 (red) showed endomucin. Colour balance settings for each channel were maintained between samples.

**Mouse Aortic ring assay:** Fkbpl<sup>+/+</sup>, Fkbpl<sup>+/neo</sup>, Fkbpl<sup>−/−</sup> mice were euthanized, and their aorta excised, washed, and stored in MCDB-131 media supplemented with 10% Pen-Strep (Gibco, UK). The aorta was stripped of all fat and connective tissue, and sliced into equal sized rings. 10 µl MCDB-131 supplemented with 10% Pen-Strep (Gibco, UK) was added to each well of a 96-well plate. 10 µl MCDB-131 supplemented with 10% Pen-Strep (Gibco, UK) was added to each well of a 96-well plate. 10 µl Matrigel (BD Biosciences, UK) was then added to this, and aortic rings were placed on top. 50 µl Matrigel diluted 1:3 with MCDB-131 media was added and the plate was incubated at 37°C for 1 h to allow the Matrigel to set. 100 µl MCDB-131 media supplemented with 10% Pen-Strep and 10% mouse serum (obtained from Fkbpl<sup>+/−</sup> mice by cardiac puncture immediately after death) was added to each well (For the VEGF treatment group, 30 ng/ml VEGF was added, and for the AD-01 treatment group, 100 nM AD-01 was added to each well) and incubated at 37°C for 10 days. Measurements and images were recorded using a Nikon Eclipse TE300 microscope and Nikon DXM1200 digital camera.

**Mouse Aortic ring immunostaining:** Sprouted aortic rings from Fkbpl<sup>+/+</sup> and Fkbpl<sup>−/−</sup> mice were washed with PBS, fixed with formalin (4% v/v) for 30 min at room temperature, permeabilized with PBS-Tween (0.1%), washed with PBlec (PBS, pH 6.8, containing in mM: 0.1 CaCl2, 0.1 MgCl2, 0.1 MnCl2, and 1% Triton-X 100), blocked with DAKO blocking buffer (Dako, North America; Catalog No, X0909) for 30 min and incubated overnight at 4°C, with anti-endomucin (Santa cruz Biotech Ltd) and anti-smooth muscle actin (Abcam) antibodies (1:1000). Aortic sections were further washed, and incubated with secondary antibodies (1:500, Alexa Fluor®488 anti-mouse and Alexa Fluor®568 anti-rat) for 2-3 h, washed with PBS-Tween and imaged on an EVOS-FL fluorescence microscope fitted
with GFP and Texas Red filter blocks. Confocal Images were captured using a Leica SP5 confocal fluorescence microscope using manufacturer settings for FITC and TRITC.

**Sponge Assay:** Method was adapted from\(^2\). Briefly, polyether sponges (Calligen Foam Ltd.) were subcutaneously implanted in Fkbpt\(^{+/neo}\), Fkbpt\(^{+/\text{+}}\) mice and their Fkbpt\(^{+/+}\) littermates as controls. Two sponges were implanted in each mouse (n= 4-5 for each group) and injected on alternate days with 10 ng murine fibroblast growth factor (βFGF, R&D Systems, Inc.). This experiment was performed on two separate occasions; total n=9-10 in each group. Sponges were removed, fixed, paraffin embedded and stained with H and E as well as endomucin as mentioned above. Vessels were blindly counted from the scanned digital images of stained sections by 2 independent assessors at x20 magnification in 10 fields per section. Two sections were made for each sponge. The average count per sponge/mouse was then plotted for each assessor.

**Tumour growth assay:** 1x10\(^6\) Lewis lung carcinoma (LLC) tumor cells were intradermally injected into the rear dorsum of male Fkbpt\(^{+/neo}\), Fkbpt\(^{+/\text{+}}\) mice and their Fkbpt\(^{+/+}\) littermates (n=5-6 in each group). This experiment was performed on two separate occasions; total n=12/group). The tumor volume and the weight of each mouse were recorded every 2 days and tumor volume was calculated as: 
\[ \frac{4}{3}\pi r^3 \] (where r = ½ GMP and GMP=\(\sqrt{\text{LengthxBreadthxHeight}}\)). The experimental endpoint was tumour volume ≥ 600mm\(^3\). Significance was determined by the log-rank test. Kaplan–Meier analysis of the data sets was applied to determine time differences to specific events, for example, time-to tumour volume ≥600 mm\(^3\). At the end of experiment the tumours (n=4/ group) were harvested and processed for IHC as mentioned above. Vasculature effects were assessed on H and E and CD31 stained tumour sections from scanned digital images as above. Vessels numbers were assessed by 2 independent assessors from 20 fields per section; each tumour was cut into 2 sections.

**Zebrafish strains and maintenance**
Zebrafish adults and embryos were maintained as previously described\(^3\). AB (wild type) and Tg (fli1:GFP) strains were used. Studies were approved by Home Office Project Licence PLL 40/3131 in the UK.

**Morpholino or peptide microinjections:** Morpholino (MO) antisense oligonucleotides were synthesised by Gene Tools LLC (USA). FKBPL UTR MO 5’-AACAAAGCCCTATTACAGACCA-3’ blocks zFkbpl protein translation. fkbpl Splice MO 5’-AGTAGTGTCTACTCCAATCACCACATCT-3’ targets the exon-2/intron-2 boundary. The respective mismatches 5’-AACGAAGCCCTATTACAGACCA-3’ and 5’-AGAAGCTTTGCTTTACCAGACACTCT-3’ were used as negative controls. cd44 UTR MO 5’-CATGGTCAGAGCTGTGTCATGCT-3’ blocks zCd44 protein translation. The respective mismatch 5’-CAAGCTCAGACCTTGACTTGGCT-3’ was used as negative control. 4 ng of MO or 1.3 pmol AD-01/Scrambled AD-01 dissolved in water were injected into one-cell Tg (fli1:GFP) zebrafish embryos (expressing the green fluorescent protein in the vasculature) near the balstodisc using a Narishige microinjector (Narishige International Ltd.) For the rescue experiments, pCDNA3.1+ hFKBPL described earlier\(^4\) was used. mRNA was synthesised using the mMessage mMachine Kit (Ambion) as previously described\(^5\) and 30 pg were co-injected with the morpholinos. Images to analyse the vasculature were captured using a Leica TCS LSI confocal microscope and Leica DFC310 FX camera.

**Statistical analysis:** Data are presented as means ± standard errors (SE). Comparisons were analysed with the Student’s t-test or one way ANOVA between two groups. Differences were considered significant when the \(P\) value was < 0.05. *\(P<0.05\), **\(p<0.01\), ***\(p<0.001\). Tukey post tests were carried out for multiple comparison.
SUPPLEMENTAL REFERENCES


SUPPLEMENTAL VIDEO FILE LEGEND

Supplemental Video: E11.5 Fkbpl+/+ transverse sections from Neural Tube level: Embryo sections were immunostained for FKBPL and Endomucin and subjected to confocal microscopy. Z-stack images demonstrate that FKBPL is clearly localised within the cytoplasm of a cell within the blood vessel lumen.
Supplemental Figure I: FKBPL RNA and protein levels upon proangiogenic cytokine stimulus: (A) VEGF and (B) bFGF did not affect FKBPL mRNA and protein levels in HMEC-1 and MDA-MB-231 cells over a range of concentrations. (C, D) VEGF stimulation over time varying time points (0.5-24h) did not alter FKBPL protein levels as well.
Supplemental Figure II: *Fkbpl*+/neo and FKBPL+/− mice generation and characterisation. (A) Schematic of chromosomal locus of the (i) WT Fkpbl allele and targeting vector utilised to target the Fkpbl locus in embryonic cells. Schematic is not to scale and FRT, LoxP & SA (splice acceptor) sites are denoted with the Fkbpl open reading frame in exon 2. Oligonucleotides utilised for mouse genotyping are depicted (a-e). (ii) Targeted Fkpbl allele demonstrating the replacement of the second exon with the LacZ reporter and Neomycin selection cassettes. (iii) Targeted Fkpbl allele following removal of the floxed neomycin selection cassette crossing heterozygote Fkbpl+/neo mice with b-actin-CRE transgenic mice. (B) Representative images (20x) of H&E stained formalin fixed, paraffin embedded murine organs demonstrate no obvious differences in the organ histology in Fkbpl+/+ and Fkbpl+/− mice. (C) Real-time quantitative PCR analysis of RNA from spleen tissue lysates of Fkbpl+/+ and Fkbpl+/− mice validating the effect of genomic knockdown on Fkbpl mRNA. Data points are mean ± SEM. n≥3. * p<0.05 (single factor ANOVA).
Supplemental Figure III Ex-vivo aortic sprouting. Aortae from (A) Fkbpl+/+ littermates showed similar sprouting to those from C57bl6 mice. (B) Fkbpl+/neo and Fkbpl+- showed similar sprouting pattern; increased levels in comparison to Fkbpl+/+ littermates. Data points are mean ± SEM. n≥3. ** p<0.01, *** p<0.001 (one-way ANOVA, Tukey multiple comparison post test).
Supplemental Figure IV Immunofluorescence staining of mouse aortic rings. (A) Fluorescence photomicrographs of mouse aortic ring grown in Matrigel stained for (i) smooth muscle (α-smooth muscle actin [green; Alexa Fluor® 488 tagged secondary antibody]) and (ii) endothelial cells (endomucin [red; Alexa Fluor® 568-secondary antibody]). Subpanel (iii) contains an overlay of green and red fluorescence. Images were captured using an EVOS-FL fluorescence microscope fitted with GFP and Texas Red filter blocks; Scale bars in A represent 400 μm. (B) Confocal fluorescence micrographs of sprouts from the same explanted mouse aorta at a higher magnification; scale bars in B represent 100 μm. Images were captured using a Leica SP5 confocal fluorescence microscope using manufacturer settings for FITC and TRITC.
Supplemental Figure V. Zebrafish orthologue to human FKBPL. (A) ClustalW2 alignment of the Homo sapiens and Danio rerio FKBPL protein sequences. Asterisk (*) and black highlight indicate positions with a fully conserved residue, colon (:) indicates conservation between groups of strongly similar properties, full stop (.) indicates conservation between groups of weakly similar properties. (B) SMART analysis of the structural domains of Homo sapiens and Danio rerio FKBPL proteins. TPR domains typical of the FKBP family are shown in yellow.