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Fibromodulin promoted *in vitro* and *in vivo* angiogenesis

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ABSTRACT

Fibromodulin (FMOD) is an extracellular matrix (ECM) small leucine-rich proteoglycan (SLRP) that plays an important role in cell fate determination. Previous studies revealed that not only is FMOD critical in fetal-type scarless wound healing, but it also promotes adult wound closure and reduces scar formation. In addition, FMOD-deficient mice exhibit significantly reduced blood vessel regeneration in granulation tissues during wound healing. In this study, we investigated the effects of FMOD on angiogenesis, which is an important event in wound healing as well as embryonic development and tumorigenesis. We found that FMOD accelerated human umbilical vein endothelial HUVEC-CS cell adhesion, spreading, actin stress fiber formation, and eventually tube-like structure (TLS) network establishment *in vitro*. On a molecular level, by increasing expression of collagen I and III, angiopoietin (Ang)-2, and vascular endothelial growth factor (VEGF), as well as reducing the ratio of Ang-1/Ang-2, FMOD provided a favorable network to mobilize quiescent endothelial cells to an angiogenic phenotype. Moreover, we also confirmed that FMOD enhanced angiogenesis *in vivo* by using an *in ovo* chick embryo chorioallantoic membrane (CAM) assay. Therefore, our data demonstrate that FMOD is a pro-angiogenic and suggest a potential therapeutic role of FMOD in the treatment of conditions related to impaired angiogenesis.

1. Introduction

The formation of new capillaries, *aka* 'angiogenesis', is an important natural process required for embryonic development, wound healing, and tumor formation [1–3]. During angiogenesis, normally quiescent endothelial cells (ECs) are stimulated to switch to an angiogenic phenotype, and undergo a cascade of events regulated by a diverse group of growth factors to complete vessel maturation [2,4,5]. Otherwise, blockage of endothelial interactions with the extracellular matrix (ECM) inhibits neovascularization *in vivo* as well as the formation of endothelial tube-like structures (TLS) *in vitro* [4,6–8], which suggests ECM molecules are also critical for angiogenesis [9–12].

Fibromodulin (FMOD) is a broadly distributed ECM protein [13] that plays an extensive role in cell fate determination, as FMOD is an integral component for maintenance of endogenous stem cell niches [14] and can directly reprogram somatic cells to a minimally proliferative, multipotent progenitor state [15]. Meanwhile, FMOD is critical for fetal-type scarless wound repair, exerts anti-scarring effects in adult wound healing, and modulates transforming growth factor (TGF)-β ligand and receptor expression [16–19]. Since FMOD-deficient mice exhibit significantly reduced blood vessel regeneration in granulation tissues during wound healing [19], FMOD may also be involved in angiogenesis.

To investigate the role of FMOD in angiogenesis, we firstly evaluated the effects of FMOD on human umbilical vein endothelial HUVEC-CS cell TLS formation. Then, we assessed FMOD's influence on cell adhesion, spreading, proliferation, and gene expression. Chick embryo chorioallantoic membrane (CAM) assay was used to confirm FMOD's effect of angiogenesis *in vivo*.

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2. Materials and methods

2.1. Cell culture

HUVEC-CS cells (passages 4–6; CRL-2873; ATCC, Manassas, VA) were cultured in DMEM supplied with 20% inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies, Grand Island, NY).

2.2. TLS formation analysis

3×10^4 cells/cm^2 HUVEC-CS cells were seeded into 6-well plates for 24 h before treatment with 3 ml fresh medium supplying 0, 2, 10 or 50 μg/ml recombinant human FMOD [15,19]. Media were changed every 3 days. On day-15, cells were fixed in 4%-paraformaldehyde in phosphate buffered saline (PBS). Five images per well, four wells per treatment were documented using an Olympus fluorescent microscope (Center Valley, PA). Images were assessed by recording dimensional and topological analyses with Image J (NIH, Bethesda, MD) [20]. Segment length of TLS is defined as the individual TLS length from one junction to the next. Calcium deposits were identified by Alizarin Red (Sigma–Aldrich, St. Louis, MO) staining [21].

2.3. Cell adhesion and spreading analysis

4-well Millicell® EZ slides (EMD Millipore, Billerica, MA) were coated with Attachment Factor (0.1% gelatin; Life Technologies) at 37 °C for 30 min. 3×10^4 cells/cm^2 HUVEC-CS cells were seeded with 0.5 ml fresh medium containing 0, 2, 10 or 50 μg/ml FMOD. After 3 h (which corresponds to the time when the collagen overlay was applied [22]), non-adhering cells were removed by a gentle wash with ice-cold PBS. After fixation, cells were stained with mouse anti-human monoclonal antibodies against vinculin (Abcam, Cambridge, CA) followed by Alexa Fluor® 594-labeled anti-mouse IgG (Life Technologies). Alexa Fluor® 488-labeled phalloidin (Life Technologies) and 4′,6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich) were used to stain actin filaments and nuclei, respectively. Six images per well, four wells per treatment were recorded and analyzed. Vinculin and actin filaments staining were documented with a confocal laser scanning microscope (CLSM; Leica Microsystems Inc., Buffalo Grove, IL).

2.4. Cell proliferation analysis

6×10^3 cells/cm^2 HUVEC-CS cells were seeded into a 96-well plate for 24 h before treatment with 200 μl fresh medium with 0, 2, 10 or 50 μg/ml FMOD. Cell proliferation was measured by Vybrant® MTT Cell Proliferation Assay Kit (Life Technologies).

2.5. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Cells were collected on day-5, -10, and -15 during TLS formation assay. RNA was extracted using RNeasy® Mini Kit with DNase treatment (Qiagen, Valencia, CA) followed by reverse transcription (RT) using iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA). qRT-PCR of *pro-α1 chain of collagen III* (*Col3α1*; prime 1: 5′-TCTTGGTCAGTCCTGTGCGGATA-3′, prime 2: 5′-AACGGATCCTGAGTCACAGACA-3′ [23]) and *tyrosine kinase receptors 2* (*Tie2*; primer 1: 5′-TTGAAGTGGAGAGAAG GTCTG-3′, primer 2: 5′-GTTGACTCATAGCTCGGACCAC-3′ [24]) was performed on a 7300 Real-Time PCR system (Life Technologies) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories). Quantitation of other genes was accessed by using TaqMan® Gene Expression Assays (Life Technologies) and SsoFast™ Probes Supermix with ROX (Bio-Rad Laboratories). Concomitant *GAPDH* was also performed in separate tubes for each RT reaction as housekeeping standard. For each gene, three separate sets of qRT-PCR analyses were performed using different cDNA templates.

2.6. CAM assay

The *in ovo* CAM assay was performed as previously described [25]. Fertilized chicken eggs (Charles River Labs, North Franklin, CT) were positioned in a horizontal position and incubated at 37 °C under 60% relative humidity in an egg incubator equipped with a turner which automatically turned eggs 6 times/day. On day-3, 5 ml albumin was withdrawn using a syringe with a 21-gage needle through the pointer end of the egg in order to allow detachment of the developing CAM from the eggshell. A rectangle window was cut in the shell as a portal of access for the CAM, and then the eggs were returned back to the incubator. On day-10, 2.0 mg/ml FMOD mixed with 30 μl 1:3-diluted growth factor reduced Matrigel (BD Bioscience, Franklin Lakes, NJ) was loaded on an autoclaved 5×5-mm polyester mesh layer (grid size: 530 μm; Component Supply Company, Fort Meade, FL), and was incubated for 45 min at 37 °C for gel formation before transplantation on the CAM. On day-13, CAMs were excised, fixed, and photographed.

2.7. Statistical analysis

Generally, data were presented as mean ± the standard error of the mean (SEM). Statistical significance was performed with OriginPro 8 (Origin Lab Corp., Northampton, MA) including one-way ANOVA, two-sample *t*-test, and Mann–Whitney analyses. A *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. FMOD promoted HUVEC-CS cell TLS formation

High-density cultured HUVEC-CS cells formed a confluent monolayer with a characteristic polygonal morphology. On day-15, several HUVEC-CS cells spontaneously sprouted and connected with each other to form TLSs on the monolayer, as previously described in adult bovine aorta ECs [26]. Compared to non-FMOD controls, FMOD administration extensively enhanced HUVEC-CS TLS formation, and induced these TLSs to establish *polygon* structures referred to as *complex meshes* (Fig. 1A), which indicates rapid cell migration [27]. FMOD significantly increased both dimensional (total length of cellular TLS network per area; Fig. 1B) and topological parameters (number of junctions, branches, and meshes per area; Fig. 1C) of TLSs. As a result, more short (0–100 μm) and less long (150–300 μm) individual TLSs (Fig. 1D, E) were observed in FMOD-treated groups (Fig. 1D and E). Interestingly, FMOD promoted TLS formation in a dose-dependent manner in a range of 0–10 μg/ml, while 50 μg/ml FMOD significantly lowered both dimensional and topological parameters of TLSs compared with that of 10 μg/ml FMOD (Fig. 1B and C). Moreover, 50 μg/ml FMOD also resulted in calcified nodule-like cluster formation (Fig. 1A and F).

3.2. FMOD accelerated HUVEC-CS cell attachment and spreading, but not cellular proliferation

FMOD significantly improved HUVEC-CS cell adhesion (Fig. 2A). For instance, 10 μg/ml FMOD resulted in approximately 82% (2.46×10^4 cell/cm^2) seeded cell attachment in the first 3 h, while

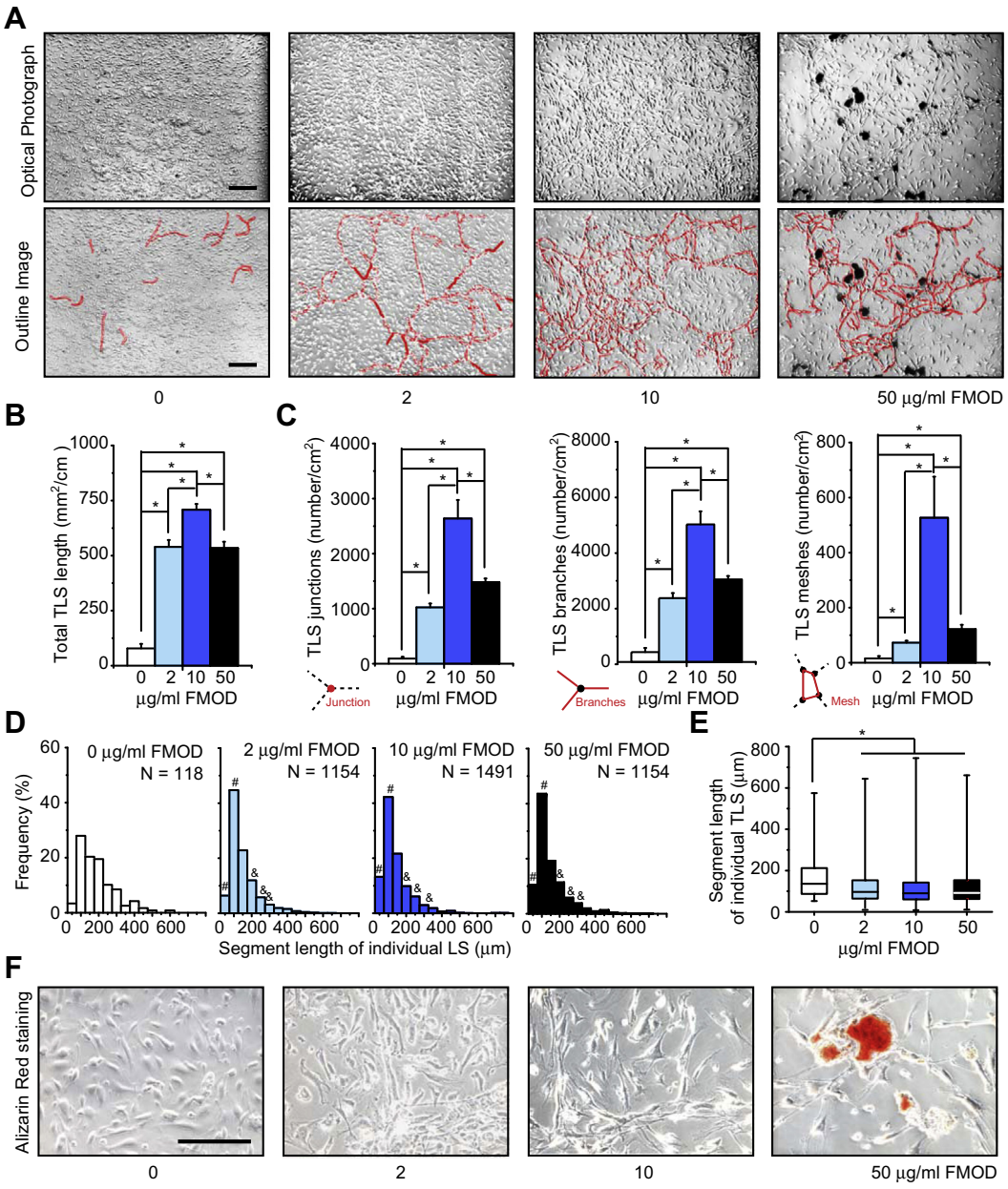

Fig. 1. FMOD promoted HUVEC-CS TLS formation *in vitro*. (A) On day-15, HUVEC-CS cells spontaneously formed TLSs (outlined in the lower panel). (B) Dimensional and (C) topological parameters of the HUVEC-CS TLS network were quantitated; $N = 4$. (D) Distribution of TLS segment length in FMOD-treated groups was compared to non-FMOD (0 μg/ml FMOD) control. # and & indicated higher and lower frequency, respectively. (E) The median (center line), 25%–75% percentiles (box) and lowest and highest values (bars) of TLS segment length were also shown and analyzed by Mann–Whiney test. (F) Alizarin Red staining demonstrated calcified cluster formation in 50 μg/ml FMOD group. Bar = 200 μm. *significant difference compared to non-FMOD control (B, C, and E).

only 46% (1.38×10^4 cell/cm^2) adhered in the non-FMOD control group during the same period. It is also worth noting that fewer cells adhered in 50 μg/ml FMOD (2.12×10^4 cell/cm^2) than in 10 μg/ml FMOD (Fig. 2A). Immune staining revealed only a small amount of vinculin-positive focal adhesion and actin stress fibers in attached non-FMOD-treated cells, while vinculin staining was co-located with actin fibers (Fig. 2B). On the contrary, FMOD significantly induced vinculin expression and actin stress fiber formation (Fig. 2B). Clear vinculin and actin stress fiber staining was observed in 2 μg/ml FMOD-treated cells (Fig 2B). In 10 μg/ml FMOD-treated cells, vinculin was intensively expressed and organized as special network throughout the cell body. Phalloidin staining also evidenced an abundant number of actin stress fibers in the cytoplasm as well as cell edge, suggesting cellular spreading and migration (Fig. 2B). Interestingly, 50 μg/ml FMOD-treated cells aggregated to form cellular clusters surrounded by aggregated vinculin and actin stress fibers (Fig. 2B). Cells were significantly more spread out when treated with 2 and 10 μg/ml FMOD compared to non-FMOD treatment (Fig. 2C). However, 50 μg/ml FMOD-treated cells adopted a "rounder" morphology in cell cluster (Fig. 2B).

Meanwhile, in order to exclude the possibility that the inducing effect of FMOD on HUVEC-CS cell TLS formation was the result of stimulating cellular proliferation, cellular viability of HUVEC-CS during culture was quantified. In comparison with non-FMOD controls, there was no significant difference in HUVEC-CS cell viability in 2 and 10 μg/ml FMOD cultivation during the entire experimental period (Supplementary Fig. 1). Significant reduction of HUVEC-CS viability was observed in long-term (day-9) cultivation with

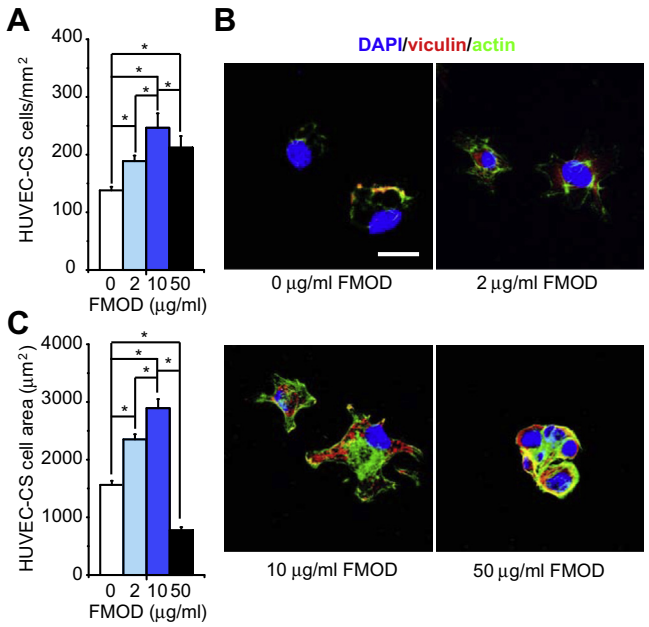

Fig. 2. FMOD promoted HUVEC-CS adhesion and spreading *in vitro*. (A) Adhered HUVEC-CS cells were calculated after 3 h cultivation. (B) Adhered cells were stained with antibody against vinculin and Alexa Fluor® 488-labeled phalloidin. Nuclei were stained by DAPI. (C) Area of adhered cells was also calculated after 3 h cultivation. *significant difference; $N = 4$. Bar = 30 μm.

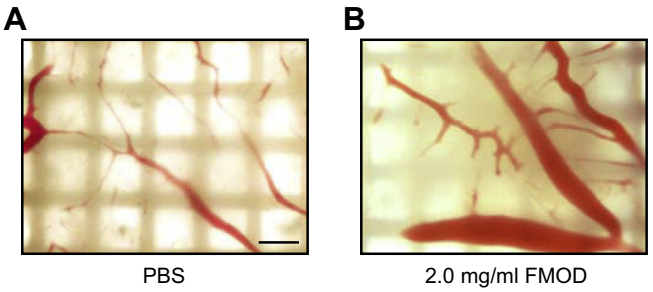

Fig. 3. FMOD promoted *in vivo* angiogenesis. Macroscopic photographs of blood vessel generation on CAM treated with (A) non-FMOD PBS control and (B) 2.0 mg/ml FMOD on day-13. Bar = 500 μm.

50 μg/ml of FMOD; however, a decrease of HUVEC-CS cell viability was not evidenced at earlier time points (Supplementary Fig. 1).

3.3. FMOD orchestrated HUVEC-CS cell gene expression in favor of angiogenesis

2 μg/ml FMOD did not notably affect type I or type III collagen expression in HUVEC-CS cells. However, by increasing the concentration of FMOD to 10 and 50 μg/ml, type I and type III collagen expression was significantly elevated (Supplementary Fig. 2A, B). FMOD also stimulated HUVEC-CS angiopoietin (Ang)-2 expression in a dose-dependent manner (Supplementary Fig. 2C). On the other hand, expression of Tie2, the predominant receptor of Ang-1 and Ang-2 signals [28], did not respond to FMOD treatment (Supplementary Fig. 2D). Interestingly, high dose (10 and 50 μg/ml) FMOD slightly inhibited Ang-1 expression in long-term (10 and 15 days) cultivation (Supplementary Fig. 3A), while the well-known angiogenesis growth factor vascular endothelial growth factor (VEGF) expression in HUVEC-CS cells was considerably upregulated by FMOD administration (Supplementary Fig. 3B).

3.4. FMOD enhanced in vivo angiogenesis

Using the *in ovo* CAM assay, we further examined the *in vivo* pro-angiogenic potential of FMOD. Macroscopic observation showed that CAMs in the non-FMOD treated group had only a few slim blood vessels on day-13 (Fig. 3A). On the other hand, a significant increase of blood vessels with large diameters was documented in CAMs treated with FMOD (Fig. 3B). Therefore, FMOD also promoted angiogenesis *in vivo*.

4. Discussion

Structurally, FMOD is a member of the small leucine-rich proteoglycan (SLRP) family, which also comprises decorin, biglycan, and lumican [13]. SLRPs are distributed in the ECM of all tissues and the thin membranes that envelop all major parenchymal organs such as pericardium, pleura, periosteum, perimysium, and adventitia of blood vessels [12]. The involvement of decorin and lumican in angiogenesis has been intensively studied [12]. For example, lumican inhibits angiogenesis by interfering with integrin $\alpha 2\beta 1$ activity and downregulating proteolytic activity associated with surface membranes of ECs [29]. Meanwhile, decorin seems to be pro-angiogenic in some early experimental settings [12,30], but it inhibits EC migration and TLS formation *in vitro*[12,22,31]. Additionally, decorin-deficiency markedly increases fibrovascular invasion and enhances the formation of blood vessels *in vivo*[32]. Additional studies demonstrated that the anti-angiogenic activity of decorin relies on blocking angiogenic signals [12,22,31,33,34], especially during pathological angiogenesis such as that in malignant vascular tumors [35,36]. It is likely that decorin plays a dual moderator role on angiogenesis.

Unlike decorin and lumican, our present results showed that FMOD significantly stimulated EC TLS formation *in vitro* as well as blood vessel generation *in vivo*. In addition, FMOD-deficiency reduced blood vessel regeneration in adult rodent cutaneous wounds [19]. In the aspect of cellular behavior, FMOD remarkably enhanced EC cell adhesion, spreading, and actin stress fiber formation. Previous studies already highlighted the importance of mechanical-tension interactions between ECs and their substrate to promote TLS formation *in vitro*[37]. It is possible that FMOD may be responsible for providing a permissive microenvironment for EC actin stress fiber formation and cell spreading during TLS organization, since FMOD plays important roles in ECM assembly, organization, and degradation [12,38,39]. Meanwhile, not only do SLRPs play structural functions in the ECM of all tissues, but they are also signaling molecules that regulate intracellular signaling cascades and determine cell fate [15,40]. Our previous studies have revealed that FMOD modulates TGF-β expression, distribution, and bioactivity in an isoform-dependent manner [19,41]. Thus, it is also possible that FMOD interferes with the activation of integrins or other cell surface receptors that initiate the formation of actin stress fibers and adhesion contacts, thus promoting cytoskeletal reorganization for spreading and TLS formation *in vitro*. On a molecular level, *in vitro* angiogenesis is correlated to ECM protein expression [9,11,26]. For instance, collagen I initiates quiescent ECs morphogenesis leading to the formation of TLSs [11,26], while collagen I and III both contribute to vessel wall stability [9,42]. In the aspect of signal transduction, we also found that FMOD enhanced HUVEC-CS cell collagen I and III expression, which correlates with the increase of TLS network organization. In addition, the Ang/Tie signaling system is essential for vessel remodeling and maturation [28]. Ang-1 is predominantly expressed in perivascular cells, and binds to Tie2 receptors resulting in promotion of EC survival signaling, the maintenance of an endothelial barrier, and a quiescent vasculature [28]. Expressed by ECs, Ang-2 acts mainly as

an antagonist for Tie2 receptor signaling by displacing the more active ligand Ang-1 from the receptor [28]. However, Ang-2 also has a direct pro-angiogenic Tie2-independent role [28]. In this study, we also showed that FMOD stimulated Ang-2 expression, slightly reduced Ang-1 level, but did not affect Tie2 transcription in HUVEC-CS cells, which suggests that FMOD mobilized quiescent ECs for angiogenesis, at least partially, *via* reducing Ang-1/Ang-2 ratios which are critical in balancing Tie2-mediated signaling and regulating vascular homeostasis and responsiveness to other angiogenic cues [28]. Furthermore, FMOD also induced VEGF expression in HUVEC-CS cells, although ECs are not the main source of VEGF. Because Ang-2 promotes angiogenesis in the presence of VEGF [28,43], FMOD thus provides a special network to mobilize quiescent ECs to undergo angiogenesis. Together, these findings strongly suggested that FMOD exhibits pro-angiogenic properties though multiple pathways.

It is also worthy to note that 50 μg/ml FMOD induced HUVEC-CS aggregation, sharply increased expression of collagen I, III and Ang-2, and eventually resulted in calcification *in vitro*. These phenomena suggest that excessive FMOD levels will result in EC dysfunction. Actually, FMOD was found to regulate tumor stromal structure, which is characterized by distorted blood vessels accompanied by elevated interstitial fluid pressure [39]. Considering the fact that massive FMOD expression was found in different malignancies [39,44,45], there seems to be a direct link between tumor progression and FMOD (and maybe other SLRPs too) [9,46].

In conclusion, we demonstrated that FMOD is capable of promoting angiogenesis *in vitro* and *in vivo*. Our findings indicate FMOD's pro-angiogenic properties are due to its powerful orchestrating of pro- and anti-angiogenic signaling. Our results provide novel insights into angiogenesis regulation, suggesting a potential therapeutic application of FMOD in wound healing and cancer therapy as well as other conditions related to abnormal blood vessel generation.

Disclosures

Drs. K. T., C. S., and Z. Z. are inventors on fibromodulin-related patent filed from UCLA. Drs. K. T., C. S., and Z. Z. are founders of Scarless Laboratories Inc., which sublicenses fibromodulin-related patents from the US Regents. Dr. Soo is also an officer of Scarless Laboratories, Inc.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.005.

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