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Interaction between angiogenin and fibulin 1: evidence and implication

Hui Zhang[1&], Xiangwei Gao[1], Chunhua Weng[1,2], and Zhengping Xu[1,2*]

[1] Research Center for Environmental Genomics, and [2] Bioelectromagnetics Laboratory, Zhejiang University School of Medicine, Hangzhou 310058, China

Angiogenin is an angiogenic factor involved in tumorigenesis. However, the mechanism of angiogenin's action remains elusive. In the present study, we identified fibulin 1, an extracellular matrix and plasma glycoprotein, as an angiogenin-interacting molecule by yeast two-hybrid screening. This interaction was further confirmed by two different approaches. First, fibulin 1 was co-immunoprecipitated with angiogenin by anti-angiogenin monoclonal antibody *in vitro*, suggesting angiogenin binds with fibulin 1 directly. Then fluorescence resonance energy transfer analysis showed that fibulin 1 interacted with angiogenin in COS-7 cells, showing that the binding could occur in a cellular context. As fibulin 1 plays an important role in cell proliferation, migration, adhesion, and stabilizes new-forming blood vessel wall, the interaction between fibulin 1 and angiogenin might underline one possible mechanism of angiogenin in angiogenesis and/or tumorigenesis.

Keywords angiogenin; fibulin 1; protein interaction; FRET; angiogenesis

Angiogenin (ANG) was originally isolated from the conditioned medium of HT-29 human colon adenocarcinoma cells based solely on its angiogenic activity [1], and was up-regulated in many types of cancers [2,3]. Animal studies showed that its monoclonal antibody (mAb), binding protein, enzymatic inhibitors, and antisense RNA suppressed tumor growth or metastasis [4–9]. Although the roles of ANG in angiogenesis and tumorigenesis have been well recognized, its mechanism of action needs to be elucidated.

ANG plays a key role in tumor angiogenesis. It activates vessel-originated endothelial and smooth muscle cells through binding with membrane proteins and undergoing nuclear translocation, and induces a wide range of cellular responses, including cell migration, invasion, proliferation, and formation of tubular structures [10,11]. Results showed that ANG bound to actin or a putative 170 kDa protein, and activated extracellular signal-regulated kinase 1/2 in human umbilical vein endothelial cells [12] or stress-activated protein kinase/c-Jun N-terminal kinase in human umbilical artery smooth muscle cells [13]. Meanwhile, exogenous ANG could translocate to the nucleus through an unknown mechanism and bind to DNA, hence enhancing rRNA transcription [14–16]. It was reported that ANG was a general requirement for endothelial cell proliferation and angiogenesis induced by various other angiogenic proteins, including acidic and basic fibroblast growth factors, epidermal growth factor, and vascular endothelial growth factor, suggesting ANG-stimulated rRNA transcription in endothelial cells might serve as a crossroad in the process of angiogenesis [15]. Recently, data showed that ANG was constitutively translocated into the nucleus of HeLa cells where it stimulated rRNA transcription, indicating that ANG also played a role in cancer cell proliferation [2].

However, there are still many knowledge gaps in clarifying the mechanism of ANG in angiogenesis and tumorigenesis. We believe that establishing a protein interaction network should help in filling these gaps. Therefore, a yeast two-hybrid technique was used to screen ANG's potential interacting molecules from a human cDNA library. In the present study, we report the identification of fibulin 1 as an ANG binding partner.

Materials and Methods

Plasmid construction

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[&] Present address: Department of Biochemistry, Zhejiang Medical College, Hangzhou 310053, China
*Corresponding author: Tel, 86-571-88208164; Fax, 86-571-88208163; E-mail, zpxu@zju.edu.cn

The full-length cDNA of *ANG* was amplified by polymerase chain reaction (PCR) (forward primer, 5′-ATATAGTCGACTCAGGATAACTCCAGGT-3′; reverse primer, 5′-ATAAGAATGCGGCCGCTACGGACGACGGAA-3′), digested with *Sal*I and *Not*I, and inserted into the corresponding sites of the vector pDBLeu (Invitrogen, Shanghai, China). This construct was named pDBLeu-ANG and subsequently used in the yeast two-hybrid screening as described below.

For *in vitro* co-immunoprecipitation analysis, *fibulin 1*, identified as an ANG binding molecule in the yeast two-hybrid screening, was amplified by PCR with primers 5′-CGGAATTCATGGCAGTGGGCTCTTTCC-3′ (forward) and 5′-AATGCGGCCGCAGGGTTAGGAAAGCAAC-3′ (reverse). Then plasmid pCMVTNT-fibulin 1 was constructed by inserting the amplified *fibulin 1* gene into vector pCMVTNT (Promega, Madison, USA), which was digested with *Eco*RI and *Not*I, and used in the *in vitro* transcription/translation system.

For molecular imaging, *fibulin 1* was amplified by PCR with primers 5′-GAAGATCTCCAAAAAAGAGGGTGGG-3′ (forward) and 5′-CGGAATTCAGCTCGACGTCTTAC-TT-3′ (reverse), and fused to cyan fluorescent protein (*CFP*) by cloning into the *Bgl*II and *Eco*RI sites of vector pECFP-C1 (Clontech, Palo Alto, USA) to generate pCFP-fibulin 1. *ANG* was amplified by PCR with primers 5′-CGGAATTCACCATGCAGGATAACTCCAGGTAC-3′ (forward) and 5′-CGGGATCCGGACGGAAAATTG-3′ (reverse), and digested with *Eco*RI and *Bam*HI. The purified fragment was inserted into pEYFP-N1 (Clontech, Palo Alto, USA) at the corresponding sites to generate pYFP-ANG.

All constructs were confirmed by DNA sequencing.

Yeast two-hybrid screening

A yeast two-hybrid screening was carried out according to the manufacturer's instructions (Invitrogen). Briefly, after passing the self-activation assessment and determining the basal expression level of the *his3* reporter gene, the bait plasmid pDBLeu-ANG was transformed into the yeast strain MaV203 together with a cDNA library prepared from human heart in the prey vector pPC86 by a lithium acetate method. Candidate clones were selected by plating cotransformants on selective medium (Trp^-, Leu^-, His^- plus 25 mM 3-amino-1,2,4-triazole) and confirmed by β-galactosidase activity analysis. Plasmids were isolated from candidate clones and retransformation assays were carried out to verify the interactions. The selected plasmids were sequenced by dideoxy sequencing. The insert sequences obtained were subjected to BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

***In vitro* co-immunoprecipitation analysis**

Fibulin 1 was *in vitro* transcribed and translated in the TNT T7 Quick coupled transcription/translation system (Promega) using pCMVTNT-fibulin1 as a template and pCMVTNT as a control. In each reaction, 3 μg plasmid DNA was added to a 50 μl reaction system containing 40 μl TNT Quick Master Mix, 2 μl [^{35}S]-methionine (3.7×10^3 Bq/mmol at 37Bq/ml; Amersham Pharmacia, Piscataway, USA). After incubation at 30 °C for 90 min, 10 μl *in vitro* translated products was mixed with 10 μg ANG (a gift from Dr. Guofu Hu at Harvard Medical School, Cambridge, USA) at 4 ºC for 2 h. Then 52.5 μl co-immunoprecipitation buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40), 20 μl Protein A agarose beads (Santa Cruz Biotechnology, Santa Cruz, USA), and 5 μg ANG mAb 26-2F were added and incubated for 2 h at 4 ºC with continuous rocking. After centrifugation at 5000 *g* for 15 s, the pellet was rinsed five times with 100 μl co-immunoprecipitation buffer each to eliminate non-specific binding. The final pellet was added with 15 μl sodium dodecyl sulfate (SDS) loading buffer, heated at 95 ºC for 5 min, and separated by SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was fixed and dried at 80 ºC under constant vacuum, and subjected to autoradiography.

Cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, USA) at 37 ºC in 5% humidified CO_2. To carry out fluorescence resonance energy transfer (FRET) analysis, glass coverslips were placed in 6-well tissue culture plates and each well was seeded with $1–3\times10^5$ cells in 2 ml complete medium. After incubation for 24 h, the transfection was carried out using PolyFect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. In the cotransfection experiment, the DNA ratio of the two plasmids was adjusted to 1 to obtain approximately equal amounts of expressed proteins.

Live imaging of proteins and FRET

Cells were imaged in complete medium at room temperature. The experiments were carried out with a confocal imaging spectrophotometer system (TCS SP; Leica, Wetzlar, Germany) attached to a Leica DMIRBE inverted epifluorescence microscope and equipped with an argon laser (458, 488, 514, and 543 nm lines) and acousto-optic tunable filter to attenuate individual visible

laser lines. In all cases a 40×NA oil immersion objective lens was applied. Quantification of FRET was determined by calculating the ratio of intensity of the fluorescence emission signals at 525 nm/475 nm while excited at the CFP excitation wavelength (458 nm).

In the photobleaching study, CFP-fibulin 1 was excited at 458 nm and detected at 470–500 nm. For ANG-yellow fluorescent protein (YFP), an excitation wavelength of 514 nm and an emission wavelength of 530–550 nm was applied. CFP-fibulin 1 and ANG-YFP images were collected at 10 s intervals for five times. After that, YFP was photobleached with the full power of the 514 nm line laser, and the emission intensities of CFP and YFP were monitored an additional five times.

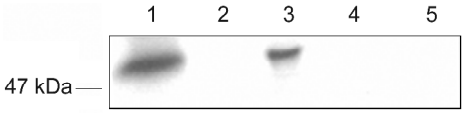

Fig. 1 Fibulin 1 interacted with angiogenin (ANG) in an *in vitro* system Fibulin 1, synthesized and labeled with [^{35}S]-Met *in vitro*, was incubated with ANG, then immunoprecipitated by anti-ANG monoclonal antibody 26-2F. The immunoprecipitated products were subjected to autoradiography. 1, the *in vitro* translated products of pCMVTNT-fibulin 1; 2, the *in vitro* translated products of vector pCMVTNT; 3, autoradiograph of immunoprecipitation products of fibulin 1 with ANG; 4, autoradiograph of immunoprecipitation products of ANG and translated products of pCMVTNT; 5, autoradiograph of immunoprecipitation products of fibulin 1 without ANG.

Results

Fibulin 1 interacted with ANG in yeast cells

To identify putative ANG-interacting proteins, a human heart cDNA library was screened by the bait protein ANG following the standard yeast two-hybrid screening procedure. Among 46 truly positive clones [17], two coded for human fibulin 1 isoform C (GenBank accession No. NM_001996) (**Table 1**), showing that fibulin 1 was an ANG binding protein in yeast cells. The longest ANG-binding fibulin 1 fragment was from amino acid 211 (corresponding to the nucleotide 707 bp in mRNA). This peptide was used in the following study.

Fibulin 1 interacted with ANG *in vitro*

Fibulin 1 was *in vitro* synthesized and labeled with [^{35}S]-Met, and subsequently incubated with ANG. The mixture was immunoprecipitated with anti-ANG mAb 26-2F in the presence of Protein A agarose beads, and the co-immunoprecipitated products were subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. **Fig. 1** clearly shows that fibulin 1 could be co-immunoprecipitated by 26-2F (**Fig. 1**, lane 3), indicating that fibulin 1 interacted with ANG *in vitro*.

Fibulin 1 interacted with ANG *in vivo* detected by FRET analysis

To confirm the binding between fibulin 1 and ANG in mammalian cellular content, we investigated the interaction between the two proteins in COS-7 cells using FRET analysis, a molecular imaging approach. Spectra of CFP-tagged and YFP-tagged proteins were first determined in cells transfected with each single construct and excited with the appropriate laser line (458 nm for CFP and 514 nm for YFP), which showed the emission spectra for CFP-fibulin 1 and ANG-YFP were rather distinct with emission peaks at approximately 475 and 525 nm, respectively [**Fig. 2(A,B)**]. For the measurement of FRET, cells expressing both CFP-fibulin 1 and ANG-YFP were excited at 458 nm. If FRET occurred between CFP-fibulin 1 and ANG-YFP, a shift in the emission spectrum of CFP (475 nm) to YFP (525 nm) would be observed. As expected, a decrease in the 475 nm emission and an increase in the 525 nm emission occurred [**Fig. 2(C)**], indicating that FRET did take place between CFP-fibulin 1 and ANG-YFP in COS-7 cells.

YFP could be excited by 458 nm light at a level approximately 30% of the intensity induced by 514 nm laser. Such "spectra cross-talk" will cause a false FRET that is dependent on the expression level of the YFP construct relative to the CFP construct. Thus, if the expression of the YFP protein was to be greatly in excess of the CFP protein, discriminating between such "spectra cross-talk" and real FRET would not be possible.

Table 1 Fibulin 1 fragments identified by yeast two-hybrid screening

Fragment	Size of insert	Corresponding first nucleotide in mRNA	Protein
1	about 1.5 kb	707	*Homo sapiens* fibulin 1, transcript variant C
2	about 1.1 kb	1043	*Homo sapiens* fibulin 1, transcript variant C

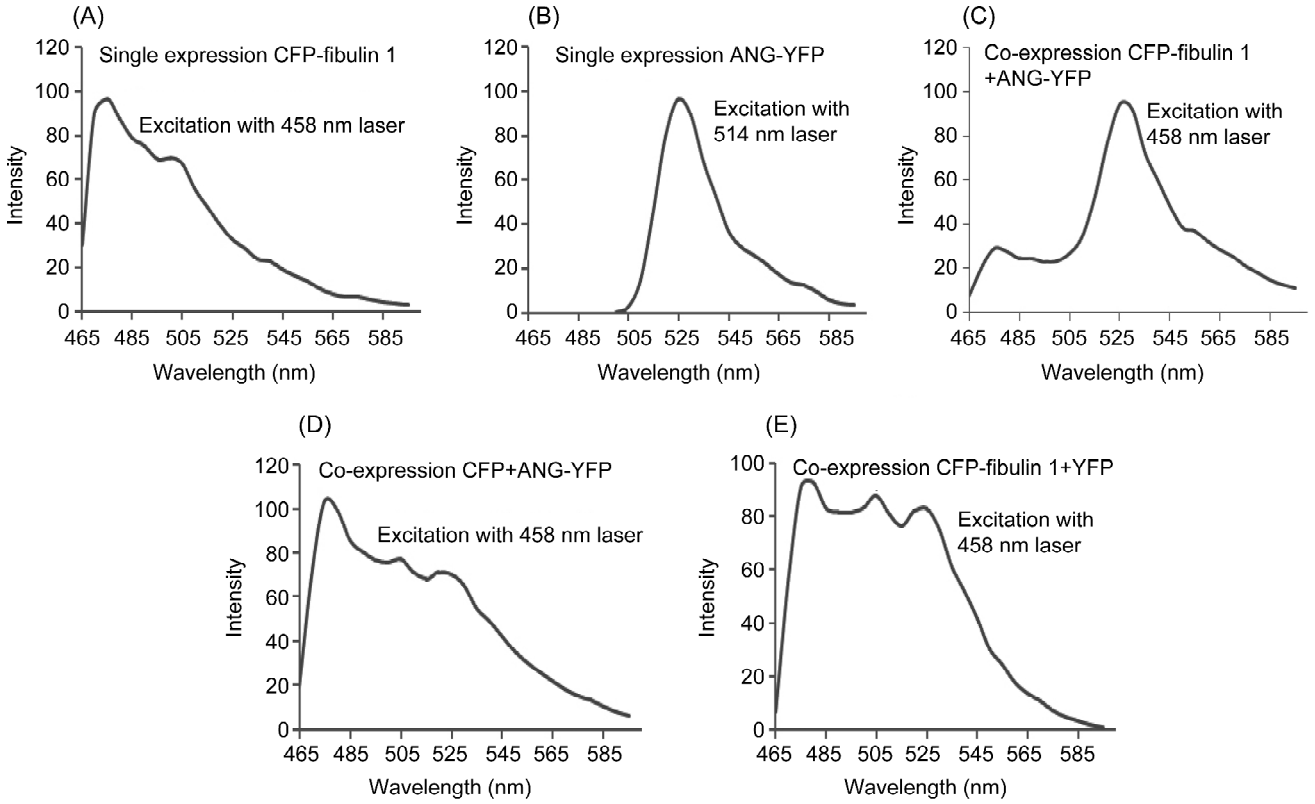

Fig. 2 Angiogenin interacted with fibulin 1 *in vivo* The fluorescence resonance energy transfer (FRET) approach was used to show the interaction between ANG and fibulin 1. COS-7 cells were transiently transfected with the relevant constructs and then scanned using a confocal imaging spectrophotometer system by 458 nm or 514 nm laser lines to excite cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP), respectively. After determining the emission spectrum of individual protein was distinct (A,B), FRET was manifested as a decrease in the 475 nm emission and an increase in the 525 nm (C). To exclude spectra "cross-talk" or the proximity effect of overexpressed proteins, FRET was analyzed between CFP-fibulin 1 and YFP or CFP and ANG-YFP. Data clearly showed that there was no significant energy transfer (D,E).

For this reason, the experimental conditions were optimized to ensure that the expression level of the YFP construct was approximate to that of the CFP construct, achieved by adjusting the plasmid ratio to 1:1 while carrying out double transfection. Furthermore, energy transfer might occur due to the physical proximity of two proteins crowded together due to overexpression. To eliminate these possibilities, CFP-fibulin 1 and YFP, or CFP and ANG-YFP were expressed in a cell simultaneously and the emission pattern of each pair was determined by spectrum scanning. Although there was an emission peak at 525 nm in both control groups [**Fig. 2(D,E)**], significant differences existed between the two control groups and cells expressing CFP-fibulin 1 plus ANG-YFP. The ratios of 525 nm to 475 nm emission intensity of the two control groups were 0.80±0.10 (n=8) and 0.81±0.12 (n=8), respectively; the ratio of the cells expressing both CFP-fibulin 1 and YFP-ANG was 2.90± 1.19 (n=8).

Photobleaching experiments confirm FRET measurements

To be more certain that the evidence for FRET described above was valid, we employed photobleaching of the YFP using a high-intensity exposure to the YFP excitation light (514 nm laser) to block energy transfer from CFP to YFP, and recorded the energy redistribution of CFP. If FRET occurs, photobleaching of ANG-YFP should lead to an increase in the 480 nm emission of CFP-fibulin1. Experimental observation showed that this was indeed the case (**Fig. 3**). Real-time imaging showed the emission intensity of CFP-fibulin 1 significantly increased following bleaching of ANG-YFP. The photobleaching results further suggested that the apparent FRET phenomenon was due to the interaction between fibulin 1 and ANG and not triggered by "spectra cross-talk" between CFP and YFP or overexpression-induced proximity of two proteins.

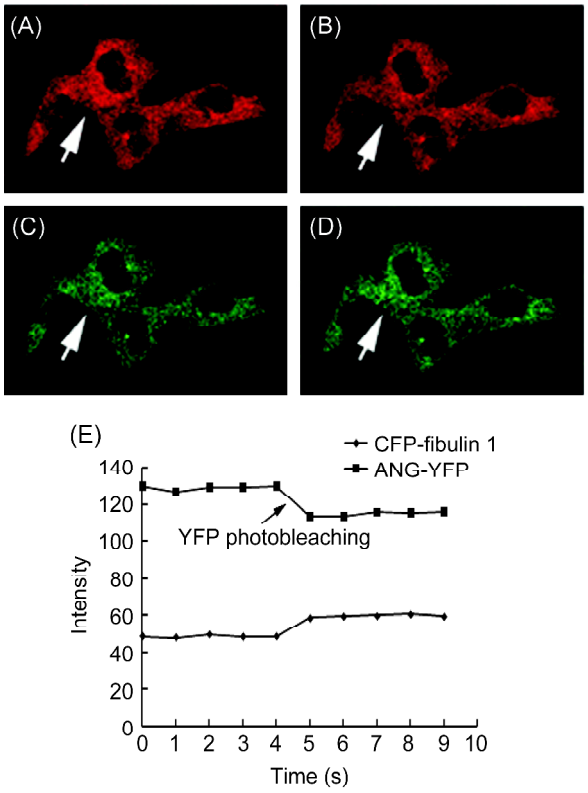

Fig. 3 Fluorescence resonance energy transfer phenomenon confirmed by photobleaching method COS-7 cells expressing both angiogenin-yellow fluorescent protein (ANG-YFP) and cyan fluorescent protein (CFP)-fibulin 1 were imaged with a confocal microscope through the YFP channel (530–550 nm) (A) and the CFP channel (470–500 nm) (C). After the YFP was photobleached with 514 nm laser at a high intensity (B), the emission signal of CFP increased (D). The dynamic change of fluorescence intensity of CFP and YFP before and after photobleaching is also presented (E).

Discussion

Fibulin 1 belongs to a six-member family of extracellular matrix (ECM) protein named fibulin [18,19]. Although its actual function remains to be explored, fibulin 1 has been suggested to be involved in cell adhesion, migration, proliferation, and malignant transformation through binding to various ECM proteins, receptors and growth factors [18–20]. Of particular interest is that fibulin 1 might be involved in angiogenesis. It was reported that fibulin 1 bound with angiogenesis inhibitor endostatin [21]. Targeted inactivation of *fibulin 1* gene in mice caused dilation and ruptures in the endothelial lining of small blood vessels [22], indicating that fibulin 1 was important in the stabilization of blood vessel walls. Therefore, it is possible that ANG induces the essential cellular processes of angiogenesis through interaction with fibulin 1.

As ANG is a key player in angiogenesis [15], the interaction between ANG and fibulin 1 (**Fig. 4**) might interfere with the normal physiological or pathological functions of fibulin 1, and affect new blood vessel formation. To obtain more information about fibulin 1 in angiogenesis, we carried out bioinformatics analysis using PathwayAssist 3.0 software (Invitrogen). Data mining suggested that the reported binding partners of fibulin 1 include fibronectin, fibrinogen, laminin, nidogen, tropoelastin, aggrecan, versican, β-amyloid precursor protein, connective-tissue growth factor, heparin-binding epidermal growth factor (HB-EGF), and the angiogenesis inhibitor endostatin. Fibulin 1 might negatively regulate cell motility and proliferation. Binding of fibulin 1 with fibronectin could generate a new anti-adhesive site, that repulses cellular interactions rather than promotes them, thus inhibiting cell adhesion to, and migration on, fibronectin [23]. Versican was described as negatively regulating cell movement [24]. Fibulin 1 might suppress cell migration through binding to aggrecan or versican. ANG is known to promote cell proliferation, motility, and adhesion [10], contrary to the

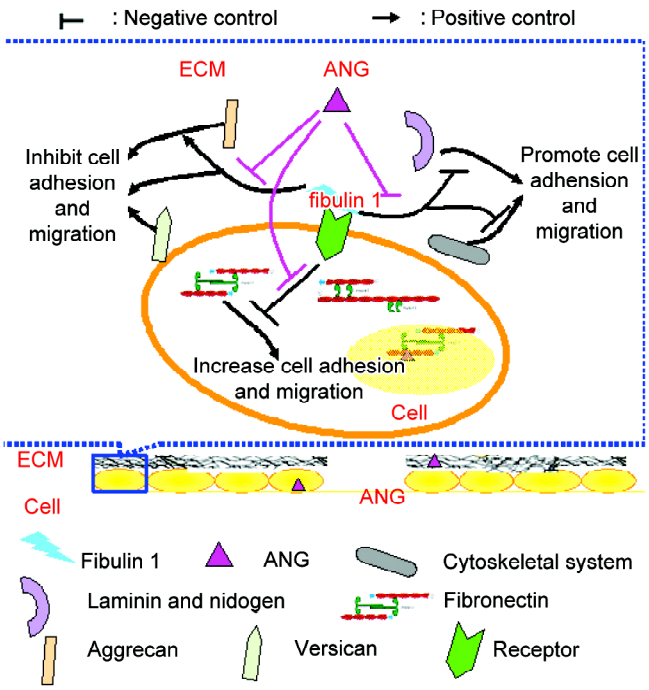

Fig. 4 Schematic illustration of the hypothetical function of the interaction between angiogenin (ANG) and fibulin 1 Based on bioinformatics analysis, we propose that one possible function of this interaction is to disrupt the suppression of fibulin 1 on cell adhesion and migration through interfering with the binding of fibulin 1 with extracellular matrix (ECM) proteins, or modulating the cytoskeletal system of the targeted cell.

functions of fibulin 1. Therefore, it is sound to speculate that one possible mechanism of action of ANG is to disrupt the suppression of fibulin 1 on these cell behaviors by interfering fibulin 1's binding to fibronectin, laminin, nidogen, versican, and aggrecan (**Fig. 4**). However, ANG was discovered to interact with cytoskeletal protein α-actinin 2 [17], and fibulin 1 was reported to control the cytoskeletal organization of endothelial cells [22]. Thus, the interaction between ANG and fibulin 1 might also modulate the cellular cytoskeletal system in favor of angiogenesis.

Taken together, we identified fibulin 1 as an interacting partner of ANG, and proposed that the interaction might partly account for the molecular mechanism underlining ANG-induced angiogenesis. It will be of high priority to clarify the pathophysiological significance of this interaction, which could be helpful in elucidating the molecular mechanism and provide a new interfering target for anti-angiogenesis and antitumor therapy.

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