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Original Article

FIZZ1 could enhance the angiogenic ability of rat aortic endothelial cells

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Abstract: Found in inflammatory zone (FIZZ1), also known as hypoxia-induced mitogenic factor (HIMF), is a secreted protein formed by 111 amino acid residues. FIZZ1 is mainly located in alveolar epithelial cells, white adipose tissue and the heart. This study aimed to explore the effects of FIZZ1 on the angiogenic ability of cultured rat aortic endothelial cells (RAECs) and the potential mechanism. The RAECs were cultured in the extracellular matrix (ECM) supplemented with 10% fetal bovine serum (FBS). Matrigel assay was used to detect the angiogenic ability of the RAECs and Agilent Rat Microarray containing 41,000 genes/ESTs was used to screen the differentially expressed genes of the RAECs after they were treated with FIZZ1 (5×10^{-9}~2×10^{-8} mol/L). The results were verified using RT-PCR method. We found that FIZZ1 markedly enhanced the angiogenic ability of RAECs (22.6 ± 2.94 vs. 19.7 ± 2.57, $P < 0.01$; 28.5 ± 3.32 vs. 19.7 ± 2.57, $P < 0.01$; 36.9 ± 5.01 vs. 19.7 ± 2.57, $P < 0.01$) in a dose-dependent manner (5×10^{-9}~2×10^{-8} mol/L). 440 genes (Gng8, Atg9a, Gdf6, etc.) were found to be up-regulated and 497 genes (Hbb-b1, Camk1g, etc.) down-regulated in the experimental group. Changes in Gng8 and Atg9a were revealed by RT-PCR. FIZZ1 could enhance angiogenesis of RAECs by up-regulating Gng8 and Atg9a.

Keywords: FIZZ1/RELMα, RAECs, angiogenesis, gene expression profiling, gene chip

Introduction

Bronchoalveolar lavage fluid from mice with experimentally induced allergic pulmonary inflammation contains a novel 9.4 kDa cysteine-rich secreted protein, which was FIZZ1 formed by 111 amino acid residues [1-6]. Its structure can be separated to 3 areas: N-Signal sequence, unfixed middle part, and unique C-ribbon formed by highly conservative Cysteine repeat motif ($^{1}CX_{l1}{}^{2}CX_{8}{}^{3}CX^{4}CX_{3}{}^{5}CX_{l0}$-$^{6}CX^{7}CX^{8}CX_{9}{}^{9}C^{10}C$). It was also called RELMα because the function area was consistent with that of other members of Resistin [7]. The Resistin family included 4 members: Relm-α/Fizz1, Relm-β/Fizz2, Resistin/Fizz3, and Relm-γ/Fizz4 [8].

Resistin-like molecules or those found in inflammatory zone (FIZZ) are a family of cysteine-rich secreted small proteins. They played an important role in the cell differentiation implicated in the pathophysiology of chronic diseases such as obesity, type 2 diabetes and helminth infections [9-14]. FIZZ1 was high expressive under the conditions of hypoxia, inflammation, peripheral blood mononuclear cells and macrophage activation [3]. Another recent study using FIZZ1 knockout (KO) mice demonstrated that the differential regulation of FIZZ1 expression is likely determined by the relative expression levels of IL-4, IL-13, and their corresponding receptors, which are differentially expressed by divergent cells [4]. In addition, FIZZ1 has been shown to stimulate myofibroblast differentiation [10, 11] and enhance the production of collagen type I and spinal muscular atrophy (SMA) in lung fibroblast cell lines [15, 16].

Angiogenesis included a cascade of intricately regulated processes occurring in growing tissues. For example, conditions of hypoxia have turned on the production of angiogenic growth factors, such as the families of vascular endothelial growth factors (VEGFs) and fibroblast growth factors (FGFs). This can be sensed by preexisting endothelial cells (ECs) in capillaries

Table 1. Primer sequences

Gene symbol	Genbank ID	Primer sequences	Product size, bp
Gng8	NM_139185	5'-CCAAGATCGCTGAGGCCCGC	168
		3'-CGCGGAAGGGATTCTCGGCG	
Atg9a	NM_001014218	5'-CCCACGGGCCCTGGAGATCA	105
		3'-CCGTGCTGGCGAACGTCCAT	
Rat-actin		5'-AGACCTTCAAGACCCCAG	136
		3'-CACGATTTCCCTCTCAGC	

and subsequently proteases are produced to dissolve the basement membrane and extracellular matrix. Therefore, ECs migrate to the direction of the stimulus, subsequently proliferate and form new vascular sprouts [17].

FIZZ1 has been shown to induce VEGF production by murine epithelial cells [18] as well as proliferation of EC, which correlating with angiogenesis in a murine model of asthma [19]. It has also been shown to induce the expression of VCAM-1 by ECs [20] which supports eosinophil trafficking by interacting with α4β1 on the cell surface of human eosinophils [21]. Since VEGF has the capacity of promoting the generation of vessel, we hypothesize that FIZZ1 may have the same capacity. In addition, it was also shown that reduction in FIZZ1 expression may contribute to the decreased angiogenesis [22]. To validate our hypothesis, we cultivate the SVARECs of rat by reorganizing the FIZZ1, and explore the effect of FIZZ1 on the angiogenic ability of cultured aortic rat aortic endothelial cells (RAECs) and the relevant potential mechanism.

Materials and methods

Cell culture

Male Wistar rats (the Department of Experimental Animals of Shandong University, Jinan, China) between 80~100 g were anesthetized with pentobarbital (30-40 mg/kg body weight, intraperitoneally). The aorta was isolated and cut into approximately 2-mm rings. 15-20 rings were placed on the bottom of 100 mm dishes and cultured in complete ECM at pH 7.45 without disturbance for 60 h. The rings were then discarded and the cells were maintained as regular endothelial cell culture. The RAECs were confirmed as endothelial in origin by using DiI-Ac-LDL direct labeling and indirect immunofluorescence microscopy using anti-rat CD31 antibody staining. The cells were maintained in ECM, supplemented with 10% fetal bovine serum (FBS, Gibco BRL), 100 U/ml penicillin (Invitrogen) and 100 μg/ml treptomycin (Invitrogen) and incubated at 37°C, 95% air and 5% CO_2. These cells were also used for up to 6-7 passages.

In vitro capillary tube formation assay in Matrigel

Tube formation assay was performed as described by Nagata, Mogi, and Walsh [24]. Twenty-four-well culture plates (Costar, Corning Incorporated) were coated with growth factor-reduced Matrigel (BD Biosciences) in a total volume of 500 μl/well and allowed to solidify for 30 min at 37°C. RAECs were trypsinized, neutralized with trypsin neutralizing solution, and resuspended in medium ECM at a density of 2×10^5/ml, and 300 μl of this cell suspension was added into each well. The wells were divided into four groups: control group, single-dose FIZZ1 group (5×10^{-9} mol/L), double-dose FIZZ1 group (10^{-8} mol/L) and 4-time-dose FIZZ1 group (2×10^{-8} mol/L). FIZZ1 were added to the appropriate well and the cells were incubated at 37°C for 12 h. Tube formation was observed under an inverted microscope (Olympus Corporation, Japan, x200), and photos were taken. Five visual fields were randomly selected from each well to count the tubes, and the average value was taken for statistical analysis.

RNA extraction

Extraction and purification of total RNA: RAECs were trypsinized and resuspended in medium ECM (supplemented with 10% FBS) at a density of 4×10^5/ml, and 2 ml of this cell suspension was added to a six-well culture plate, and then to the FBS-free medium after the cells were incubated at 37°C for 24 h. The same amount of 5×10^{-9} mol/L FIZZ1 or saline was added to the six-well plate, separately. Total RNA was extracted from cells to which FIZZ1 or saline was added.

RNA labeling and array hybridization

Sample labeling and array hybridization were performed according to the Agilent One-Color

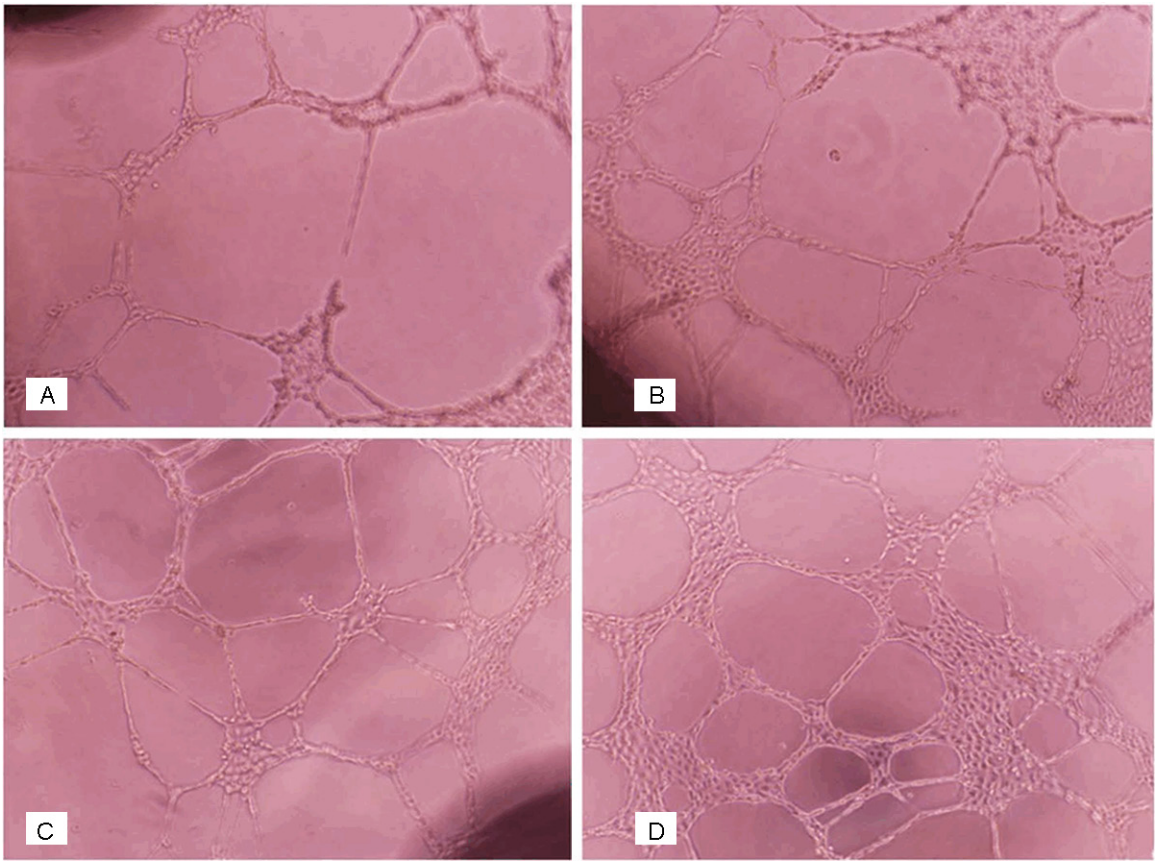

Figure 1. A: Control group. B: 2 x 10^{-8} mol/L FIZZ1 group. C: 10^{-8} mol/L FIZZ1 group. D: 5 x 10^{-9} mol/L FIZZ1 group. In vitro capillary tube formation assay in Matrigel for each group (x200). Five visual fields were randomly selected from each well to count the tubes, and the average value was taken for statistical analysis. The average tubes in control groups, 5 x 10^{-9} mol/L FIZZ1 groups, 10^{-8} mol/L FIZZ1 groups, 2 x 10^{-8} mol/L FIZZ1 groups were 19.7, 22.6, 28.5 and 36.9, respectively. There were statistically significant difference between the experimental and the normal control groups. (22.6 ± 2.94 vs. 19.7 ± 2.57, P < 0.01; 28.5 ± 3.32 vs. 19.7 ± 2.57, P < 0.01; 36.9 ± 5.01 vs. 19.7 ± 2.57, P < 0.01).

Table 2. The number of the tubes in each group ($\bar{x}$ ± S, n = 4)

Group	Number of the tubes (one/HP)
Control	19.7 ± 2.57
5 × 10^{-9} mol/L FIZZ1	22.6 ± 2.94*
10^{-8} mol/L FIZZ1	28.5 ± 3.32*
2 × 10^{-8} mol/L FIZZ1	36.9 ± 5.01*

*P < 0.05 (compared with the control group).

Microarray-Based Gene Expression Analysis protocol (Agilent Technology). Briefly, 1 μg of total RNA from each sample was linearly amplified and labeled with Cy3-dCTP. The labeled cRNAs were purified by RNAeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured by NanoDrop ND-1000. 1 μg of each labeled cRNA was fragmented by adding 11 μl 10 x blocking agent and 2.2 μl of 25 x fragmentation buffer, and then heated at 60°C for 30 min. Finally 55 μl 2 x GE hybridization buffer was added to dilute the labeled cRNA. 100 μl of hybridization solution was dispensed into the gasket slide and assembled to the gene expression microarray slide. The slides were incubated for 17 h at 65°C in an Agilent hybridization oven. The hybridized arrays were washed, fixed and scanned using the Agilent DNA Microarray Scanner (part number G2505B).

Real-time quantitative PCR analysis

To verify gene changes identified by microarray analysis, we used quantitative real-time PCR (RT-PCR) as previously described. Atg9a and Gng8 were randomly selected from the differ-

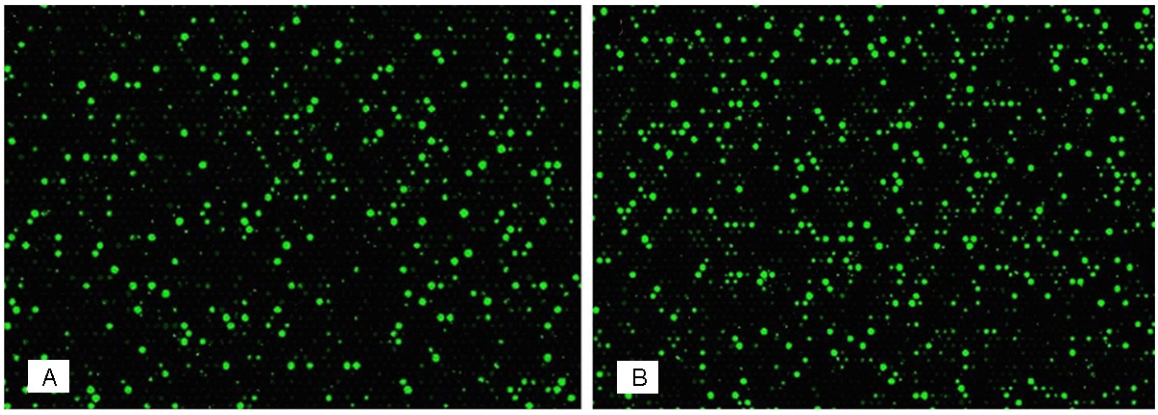

Figure 2. The hybridized arrays were scanned using the Agilent DNA Microarray Scanner (part number G2505B) and significant differences were found. A was the hybridized array from the control group; B was the hybridized array from the experimental group.

entially expressed genes screened. The techniques used were identical to those previously described. Using TRIzol (Invitrogen, USA), we extracted total cellular RNA from the experimental (5 x 10^{-9} mol/L FIZZ1) and control groups. RNA samples were reverse-transcribed using the ReverTraAce® qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's protocol. RT-PCR was performed with 10 μl SYBR® Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) in a 20 μl reaction mix containing cDNAs (2 μl), 6 μl nuclease-free water, 1 μl each (forward and reverse) primer. Primer sequences were shown in **Table 1**. Amplification was performed in a thermal cycler (ABI PRISM® 7700; Applied Biosystems, Foster City, CA) under the following conditions: initial heating at 95°C for 60 s, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and then final melting curve analysis.

Statistical analysis

The results from tube formation assay and RT-PCR were presented as Means ± SD. The treatment effects were analyzed using SPSS 15.0. Data were analyzed by analysis of variance (ANOVA), and the difference between the groups was analyzed by t-test. *P* values < 0.05 were considered to be statistically significant.

Agilent Feature Extraction software (version 10.7.3.1) was used to analyze the acquired array images. Median normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). After median normalization of the raw data, genes that at least 2 out of 2 samples have flags in Detected ("All Targets Value") were chosen for further data analysis. Differentially expressed genes were identified through fold change filtering. Hierarchical Clustering was performed using the Agilent GeneSpring GX software (version 11.5.1). Gene Ontology (GO) analysis and Pathway analysis were performed in the standard enrichment computation method.

Results

FIZZ1 enhanced the angiogenic ability of RAECs

In a dose-dependent manner(5 x 10^{-9}~2 x 10^{-8} mol/L), FIZZ1 markedly enhanced the angiogenic ability of RAECs (**Figure 1**). There was statistically significant difference between the experimental and the normal control groups. ($P < 0.05$) (**Table 2**).

Global gene expression profiles

In order to evaluate the difference of gene expression in the control and experimental groups, we conducted microarray analysis using the Agilent DNA Microarray Scanner (part number G2505B), and found significant differences (**Figure 2**). Median normalization and subsequent data processing were performed using the Gene Spring GX v11.5.1 software package (Agilent Technologies). After median normalization of the raw data, genes that at least 2 out of 2 samples have flags in Detected

Table 3. Genes representing upregulated expression in FIZZ1 cultures

GenBank accession	Gene symbol	Fold change (Treat/Control)	Description
NM_001107354	Hist1h2an	5.7152147	Histone cluster 1, H2an (Hist1h2an)
NM_001135046	Arpp-21	5.677911	Cyclic AMP-regulated phosphoprotein (Arpp-21)
NM_001002813	Ctsql2	5.3080893	Cathepsin Q-like 2 (Ctsql2)
NM_031343	Slc6a2	4.9812746	Solute carrier family 6 (neurotransmitter transporter)
NM_001014779	Pcdhb8	4.9663377	Protocadherin beta 8 (Pcdhb8)
NM_032070	Hmga2	4.871653	High mobility group AT-hook 2 (Hmga2)
NM_001007235	Itpr1	4.7131433	Inositol 1, 4, 5-triphosphate receptor
NM_001014218	Atg9a	4.6005354	ATG9 autophagy related 9 homolog A (Atg9a)
NM_139185	Gng8	4.3666644	Guanine nucleotide binding protein (G protein)
NM_198049	Slc10a6	4.2526517	Solute carrier family 10 member 6 (Slc10a6)

Table 4. Genes representing downregulated expression in FIZZ1 cultures

Genbank accession	Gene symbol	Fold change (Treat/Control)	Description
BF290343	-14.0022	UNKNOWN	BF290343 EST454934 Rat Gene Index
NM_001014059	-11.245	RGD1304952	Similar to RIKEN cDNA C530028021 gene (RGD1304952)
NM_198776	-8.75434	Hbb-b1	Beta-glo (MGC72973)
NM_182842	-7.93352	Camk1g	Calcium/calmodulin-dependent protein kinase IG (Camk1g)
NM_013088	-7.78243	Ptpn11	Protein tyrosine phosphatase, non-receptor type 11 (Ptpn11), transcript variant 2
NM_001013853	-7.64786	LOC287167	Globin, alpha (LOC287167)
NM_001000020	-7.22534	Olr1454	Olfactory receptor 1454 (Olr1454)
NM_001025002	-7.21841	LOC310926	Hypothetical protein LOC310926 (LOC310926)
XM_001059821	-7.17914	Elf4	PREDICTED: E74-like factor 4 (ets domain transcription factor) (Elf4)
M83679	-7.09958	Rab15	Sprague-Dawley (clone LRB9) RAB15 Mrna

("All Targets Value") were chosen for further data analysis. We identified 937 differentially expressed transcripts in FIZZ1 through fold change filtering. Among the identified genes, 497 transcripts were down-regulated and 440 up-regulated in FIZZ1 (**Tables 3** and **4**).

mRNA analysis of control and FIZZ1 group

To monitor the transcriptional regulation of FIZZ1 proteins, we performed real-time quantitative PCR analysis using primers designed based on the GenBank sequences of Rat-actin, Gng8 and Atg9a. Rat-actin was used as an internal RNA loading control. Our data showed significant difference in the up-regulation of Gng8 and Atg9a between the control and FIZZ1 groups (**Figure 3**).

Discussion

It is reported that FIZZ1 possesses functions such as stimulating the generation and movement of pulmonary artery smooth muscle cells, contracting vessels and stimulating myofibroblastic differentiation [23, 24], and it plays a significant role in pathogenic mechanism of many diseases such as pulmonary fibrosis, asthma, hypoxia, silicosis, and chronic obstructive pulmonary disease (COPD). However, whether it can promote the generation of endothelial cells has not been reported. Tong et al. [15, 17] showed that FIZZ1 had the functions of stimulating VCAM-1 and VEGF, which can stimulate vessel generation and affect the receptors on endothelial cells, activate P13K and phosphorylate Akt which was key element in the signaling pathway of angiogenesis. They reported that FIZZ1 can further induce VEGF expression through PI3K-AKt-NF-κB signaling pathway, PI3K-depressor (LY294002) can only partially restrain the proliferation of pulmonary vascular smooth muscle cells and the migration of FIZZ1. In addition, Yamaji et al [25] found that RELMα can promote the generation of pulmo-

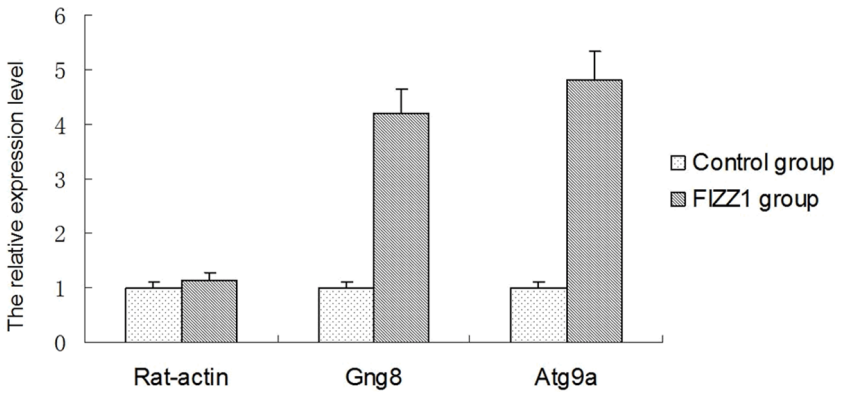

Figure 3. mRNA expressions of Rat-actin, Gng8 and Atg9a in two groups. Total RNA was extracted from RAECs and RAECs with FIZZ1 at the end of 12 h after drug administration. Results are expressed as fold relative to mRNA levels in RAECs after FIZZ1 treated with control group.*P < 0.05, compared to respective control.

nary microvascular endothelial cells, while anti-VEGF can only partly inhibit angiogenesis. Therefore, multiple ways to stimulate the generation of vessels may exist, it is possible that FIZZ1 could stimulate the aortic endothelial cells of new blood vessels and the mechanism is complicated and diversified.

The results of this study showed that FIZZ1 can obviously promote the formation of RAECs newborn tube. After RAECs are treated with the reorganized FIZZI at the concentration of 5 x 10^{-9}~2 x 10^{-8} mol/L, FIZZ1 increased the angiogenic ability of RAECs obviously. It means that FIZZ1 can significantly promote the vessel generation of RAECs.

To further explore the mechanism underlying the stimulation of vessel generation by FIZZ1, microarray analysis and RT-PCR were used to analyze the control and experimental groups. The results showed that RAECs of the experimental group are quite different from those of the control group in number after the expression of FIZZ1. The analysis result of Gene Spring GX v11.5.1 showed that over 440 genes (Gng8, Atg9a, Gdf6, etc.) existed. Atg9a was known as a transmembrane protein and the molecule of N and C-terminal transmembrane 6 participated in signal transduction of the membrane outside [26]. Gng8 participated in the multi-cell signal transduction through the cell membrane G-protein phosphorylation and dephosphorylation [27]. Gng8 and Atg9a may be associated with the ability of the FIZZ1 to promote RAECs *in vitro* angiogenesis.

To sum up, our research explored the mechanism and the ability of FIZZ1 to stimulate SVAREC vessel generation. It was found that FIZZ1 can promote the vessel generation ability of RAECs through the up-regulated expression of Gng8, Atg9a and other genes besides P13K/Akt pathway. Whether the increased expression of Gng8, Atg9a and other genes can promote angiogenesis ability of specific signal transduction pathways in RAECs cultued with FIZZ1 still needs further research.

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Disclosure of conflict of interest

None.

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