J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-016-1574-y
36(2):243-248,2016

Knockdown of Ezrin Suppresses the Migration and Angiogenesis of Human Umbilical Vein Endothelial Cells *In Vitro*[*]

Liang-ping ZHAO (赵良平)[1], Lei HUANG (黄 磊)[1], Xun TIAN (田 训)[1], Feng-qi LIANG (梁逢奇)[1], Jun-cheng WEI (魏军成)[2], Xian ZHANG (张 娴)[1], Sha LI (李 莎)[1], Qing-hua ZHANG (张庆华)[1#]
[1]*Department of Gynecology & Obstetrics, Wuhan Central Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430014, China*
[2]*Department of Gynecology & Obstetrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*

Summary: Progressive tumor growth is dependent on angiogenesis. The mechanisms by which endothelial cells (ECs) are incorporated to develop new blood vessels are not well understood. Recent studies reveal that the ezrin radixin moesin (ERM) family members are key regulators of cellular activities such as adhesion, morphogenetic change, and migration. We hypothesized that ezrin, one of the ERM family members, may play important roles in ECs organization during angiogenesis, and new vessels formation in preexisting tissues. To test this hypothesis, in this study, we investigated the effects of ezrin gene silencing on the migration and angiogenesis of human umbilical vein endothelial cells (HUVECs) *in vitro*. HUVECs were transfected with plasmids with ezrin-targeting short hairpin RNA by using the lipofectamine-2000 system. Wound assay *in vitro* and three-dimensional culture were used to detect the migration and angiogenesis capacity of HUVECs. The morphological changes of transfected cells were observed by confocal and phase contrast microscopy. Our results demonstrated that the decreased expression of ezrin in HUVECs significantly induced the morphogenetic changes and cytoskeletal reorganization of the transfected cells, and also reduced cell migration and angiogenesis capacity *in vitro*, suggesting that ezrin play an important role in the process of HUVECs migration and angiogenesis.
Key words: ezrin; RNA interference; human umbilical vein endothelial cell; migration; angiogenesis

The formation of new capillary blood vessels through the morphological change of endothelial cells (ECs), is known as angiogenesis, which is involved in many important developmental events, such as wound healing, tumor development, and embryonic development[1]. Angiogenesis includes several rate-limiting steps during aggressive tumor growth and tumor metastases[2]. The newly formed vessels not only provide metabolic support for expanding tumor population but also facilitate tumor cells entering the systemic circulation[3]. Circulating tumor cells then exploit EC receptors in distal vascular beds to promote their arrest and retention in target organs[4]. The implication of angiogenesis in tumor proliferation suggests that suppressing angiogenesis is an effective way to inhibit tumor progression. Angiogenesis is intricately regulated by a number of factors, including extracellular matrix (ECM), growth factors, membrane-bound proteinases, and integrin. These factors lead to cytoskeletal rearrangement, which delicately orchestrates the various steps of angiogenesis, including EC proliferation, branching, sprouting, and lumen formation[5]. Cells migrate by rearranging of actin cytoskeleton, which occurs in five steps, forming new lamellipodia, adhering to the substratum at the front of the cell, detaching from the substratum at the tail of the cell, and retracting their tails[6]. The actin cytoskeleton apparently plays a critical role in regulating the complex series of signaling events of ECs shape changes during migration and angiogenesis.

The ezrin radixin moesin (ERM) family members, including ezrin, radixin and moesin, act as linkers between the plasma membrane and the cortical actin cytoskeleton[7]. In the majority of eukaryotic cells, ERM proteins are involved in many physiologic functions including regulation of actin cytoskeleton, control of cell shape, adhesion, motility and modulation of signal transduction pathways[8]. Ezrin, which is considered the prototype member of the ERM protein family, was first characterized as a component of microvilli in a variety of cell types. To date, a large spectrum of cellular functions may directly or indirectly depend on ezrin. Ezrin is both a key plasma membrane-cytoskeleton crosslinker and the binding partner of a plethora of molecules with diverse functions, such as: ICAM-1, -2, -3, CD43, CD44, RhoGDI, calpain[9]. It plays a key role in the actin based cellular functions as consequence for cell locomotion, which is of importance in angiogenesis.

In the present study, we investigated the mechanisms of ezrin involved in the migration and angiogenesis of ECs. Our study focused on the role of activation of ezrin in mediating the changes of cell morphology and mobility as well as in alteration of capability of EC angiogenesis *in vitro*. Here we report silencing of ezrin may reduce the migration and angiogenesis of ECs.

Liang-ping ZHAO, E-mail: Zhaoliangping@foxmail.com
[#]Corresponding author, E-mail: Zhangqh66@qq.com
[*]This project was supported by grants from the National Natural Science Foundation of China (No. 81101950) and Research Project Foundation of Health and Family Planning Commission of Wuhan City (No. WX15C37).

1 MATERIALS AND METHODS

1.1 Cell Culture and Reagents

The HUVECs line was purchased from Wuhan University, China. HUVECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) at 37°C in 5% CO_2. The mouse monoclonal antihuman ezrin antibody, rabbit monoclonal antihuman β-actin antibody, and *Bam*HⅠ and *ECO*RⅠ were purchased from Santa Cruz Biotechnology (USA). Trizol reagent was purchased from Gibco (USA). Oligonucleotides were procured from the Shengong Biotechnology (Shanhai, China). Rabbit anti-mouse IgG alkaline phosphatase conjugate was obtained from Zhongshan Biotechnology (Beijing, China). Collagen type-Ⅰ and cell culture reagents and other chemical materials were purchased from Sigma (USA). Plasmid (pSIREN-DNR-DsRed-Express) was purchased from Clontech (USA).

1.2 Preparation of Plasmid and Transfection of HUVECs

Two independent short hairpin RNA (shRNA) sequences with different inhibition efficiencies targeted for human ezrin were chosen: Eai, CCTGGAAATGTATGGAATCAA; and Ebi, CCCACGTCTGAGAATCAACAA. DNA oligos containing the target sequence were synthesized, annealed and inserted into the expression vector (pSIREN-DNR-DsRed-Express). The accuracy of the inserted sequences of the recombinants was verified by restriction enzyme analysis and sequencing. Both constructs demonstrated significant knockdown of ezrin. Among them, the Eai construct consistently achieved lower ezrin depression (>85%) and was used for the majority of experiments. The plasmid containing shezrin was amplified in *E.coli* DH 5α strain. The protocols for plasmid preparation and purification were from Molecular Cloning: A Labrotory Manunal[10]. HUVECs (60% confluent/100 mm) were transfected with empty vector (pSIREN-DNR-DsRed-Express) or pSIREN-DNR-DsRed-Express-shezrin. Lipofectamine (Gibco-BRL Life Technologies, USA) was used as a carrier to transfect cells that had been serum-starved for 1 h and then incubated with vectors for 4 h at 37°C in a humidified atmosphere of 5% CO_2. To permit cell recuperation, the mixture was replaced by complete medium containing 10% FCS and incubated overnight. The levels of ezrin in the transfected cells were detected by immunodection of cell lysates with an anti-ezrin epitope monoclonal antibody (SantaCruz Biotechnology, USA) and semi-quantitated reverse transcription-polymerase chain reaction (RT-PCR).

1.3 RNA Isolation and RT-PCR

Total RNA was isolated from 1×10^7 HUVECs by using Trizol™ reagents. Two μg RNA was used for cDNA synthesis by reverse transcription (RT). The RNA samples were incubated at 70°C for 5 min with 0.5 μg oligo-deoxythymidine primers in a final volume of 10 μL, and then incubated at 37°C for 60 min. RT was performed in a 25 μL reaction containing 1.25 mmol/L deoxynucleotide triphosphate, 200 U Moloney murine leukemia virus reverse transcriptase and 1×buffer. Obtained cDNAs were amplified by using specific primers. Ezrin (sense primer: 5'-GCAGAATTCATGCCGAAACCAATCAATGTC-3', and anti-sense primer: 5'-GATCTCGAGTTACAGGGCCTCGAACTCGT-3') and GAPDH (sense primer: 5'-ACGGATTTGGTCGTATTGGG-3' and anti-sense primer: 5'-TGATTTTGGAGGGATCTCGC-3') primers were used in the RT-PCR reaction. The PCR reaction system consisted of 5 μL of cDNA, 0.2 mmol/L deoxynucleotide triphosphate, 1.25 mmol/L $MgCl_2$, 2.5 U Taq polymerase, 1× buffer, and 10 μmol/L primers. The PCR profile was 94°C for 30 s, 59°C for 1 min, 72°C for 90 s, for 28 cycles, followed by extension for 10 min at 72°C. The PCR products were electrophoresed on 1.5% agarose gels (Invitrogen, USA), including 0.1 μg/mL ethidium bromide (EB). The amount of mRNA was semiquantitated by measuring the relative ratio against the amplified GAPDH with equal amount of cDNA.

1.4 Protein Extraction and Western Blot Analysis

Thirty-six h after transfection, HUVECs were lysed in Laemmli buffer and centrifuged at 10 000 r/min for 5 min at 4°C. The supernatants were saved as nuclear extracts. Samples were resolved by SDS-PAGE on 12% polyacrylamide gels and electrotransferred onto 0.2 μm nitrocellulose membranes. After a blocking with 5% nonfat powdered milk in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) at 37°C for 1 h, the membranes were probed with the mouse monoclonal antihuman ezrin antibody (1:1000) and the rabbit monoclonal antihuman β-actin antibody (1:1000) respectively at 4°C overnight. After three times of washes with TBST, the membranes were incubated with secondary alkaline phosphatase-conjugated antibody at 37°C for 1 h. Nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) was used to detect the hybridized protein. Immunoblots were exposed to Kodak Image films, which were further scanned to quantify band intensities by using Biomax image analysis software. Protein extracts were loaded equivalently to detect the expression level of β-actin. Protein concentrations of extracts were determined in the Bio-Rad protein assay system with bovine serum albumin (BSA) as control.

1.5 Confocal Microscopic Analysis of Actin Filaments

The confocal microscopy was used to analyze actin filaments of HUVECs. The protocol was in accordance with that described by Peng *et al*[11]. Actin filaments were visualized by rhodamine-labeled phalloidin (Molecular Probes, USA). HUVECs transfected with ezrin-shRNA or empty vector, seeded on plates, were fixed in 3.7% formaldehyde for 10 min at room temperature. Each plate was placed in a glass Petri dish and incubated with a solution of 0.1% Triton X-100 in phosphate-buffered saline (PBS, pH 7.4) for 5 min. HUVECs were incubated with the primary monoclonal antibody against rhodamine-labeled phalloidin for 1 h at room temperature, followed by an additional incubation for 45 min with the appropriate secondary fluorescent-conjugated antibody. To reduce nonspecific background staining, fixed cells were pre-incubated with PBS containing 1% BSA for 30 min prior to adding the first antibody. Cells were washed three times during each antibody addition. Confocal laser scanning microscopy was performed by using a BioRad MRC1024 confocal imaging system (UK). HUVECs' morphology was observed under an OLYMPUS phase contrast microscope. HUVECs transfected with ezrin-shRNA or empty vector were photographed 16 h later.

1.6 Wound-closure Assay

HUVECs 24 h after transfection (ezrin-shRNA or empty vector) were seeded into 35-mm dishes at 1.5×10^5 cells/cm^2 and allowed to grow to 90% confluence in the

media with 0.1% BSA for 24 h[12]. The confluent cell monolayers were scraped with a sterile pipette tip with a constant diameter. For each dish, three to five wounds were performed, and three sites of regular wounds were selected and marked. Wounded monolayers were then washed three times with PBS to remove cell debris. Cells were permitted to migrate into the area of clearing for 24 h. Immediately after wounding and at the end of the experiment (after 24 h), wounds were photographed and semiquantitative measurements were made of control and treated wounds. A mean wound width was determined, and the average percent wound closure was calculated in a way as described previously[12].

1.7 *In Vitro* Angiogenesis Model

In vitro angiogenesis assays were performed in 3-dimensional fibrin matrixes as described previously (2 mg/mL collagen type Ⅰ 3 vol.; 10×EBSS 1 vol.; 0.1 N NaOH 1 vol.; DMEM 3 vol.; FCS 2 vol.)[12]. The cells carrying ezrin-shRNA or empty vector (1×10^4 cells/well) were suspended in the recommended medium, added to each well of Matrigel-coated 6-well plates, and the gels were incubated at 37°C in 5% CO_2 at 100% humidity. The formation of tubular structures of ECs in the 3-dimensional fibrin matrix was observed and photographed with phase-contrast microscopy on the 4th day of culture, and the mean number of cordlike structures of 6 randomly chosen microscopic fields (7.3 mm^2/ field) was recorded.

1.8 Statistical Analysis

Student's *t* test was used to compare data between two groups. Values are expressed as $x\pm s$ of at least triplicate samples. $P<0.05$ was considered statistically significant.

2 RESULTS

2.1 Ezrin Expression in Ezrin-shRNA Transfected HUVECs

Previous studies showed that the expression of ezrin peaked at 36 h post transfection[13]. We also determined the levels of ezrin expression at this time point. As expected, the level of ezrin mRNA was significantly reduced in the ezrin-shRNA transfected cells when compared with that in empty vector transfected cells (29.2±8.07 and 55.86±10.72, $P<0.05$). Western blotting showed that the relative expression level of ezrin protein was 24.84±6.89 and 50.56±8.78 in ezrin-shRNA transfected cells and empty vector transfected cells, respectively ($P<0.05$), which was in accordance with the expression of ezrin mRNA (fig .1A and 1B).

2.2 Effects of Ezrin Silencing on the Endothelial Actin Cytoskeleton and Cell Morphology

Confocal and phase contrast microscopy revealed that HUVECs transfected with empty vectors had stronger stress fibers, which were visualized by staining with rhodamine-phalloidin (fig. 2A) as described previously[14, 15]. In contrast, cells transfected with ezrin-shRNA had few actin stress fibers. Meanwhile, it was obvious that the number and diameter of stress fibers were decreased, and lamellipodia extensions reduced when the ezrin expression was inhibited. It was suggested that ezrin played an important role in regulating the organization of actin stress fibers. The morphological changes induced by down-expression of ezrin in HUVECs were observed by light microscopy. The cells transfected with ezrin-shRNA showed regular spindle shapes and lower levels of focal adhesion and of lamellipodia and filopodia formation (fig. 2B). Taken together, these data indicated that inhibition of ezrin expression can change the morphology of ECs by reorganization of actin cytoskeleton.

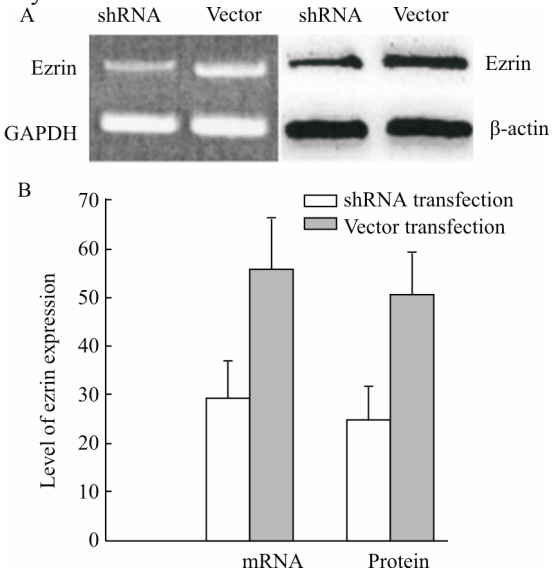

Fig. 1 The expression levels of ezrin mRNA and protein in the cells transfected with empty vectors or ezrin-shRNAs at 36 h post-transfection

A: semiquantitated RT-PCR and Western blot analyses of the expression of ezrin with GAPDH (level of mRNA) and β-actin (level of protein) at internal controls. The experiment was repeated three times. B: the bar graph of the relative expression of ezrin at mRNA and protein levels. The ezrin expression in ezrin-shRNA transfected cells was significantly lower than that of empty vector transfected cells (mRNA: 29.2±8.07 *vs*. 55.86±10.72, $P=0.026$, $n=3$; protein: 24.84±6.89 *vs*. 50.56±8.78, $P=0.016$, $n=3$).

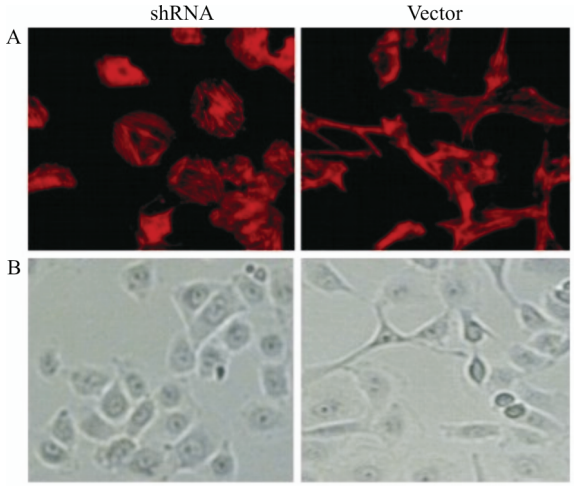

Fig. 2 Effects of ezrin-shRNA transfection on the endothelial actin cytoskeleton and cell morphology

A: F-actin was visualized by rhodamine-phalloidin staining (×600). HUVECs transfected with empty vectors had more stress fibers. In contrast, the number and diameter of stress fibers were reduced obviously in cells transfected with ezrin-shRNAs. B: HUVECs morphology was observed with phase contrast microscopy (×600). The cells transfected with ezrin-shRNAs showed lower levels of focal adhesion and lamellipodia as well as filopodia formation.

2.3 Effects of Ezrin Suppression on the Motility of HUVECs

To investigate whether ezrin was involved in the endothelial migration, confluent and quiescent monolayers of HUVECs were wounded. Recovery of these monolayer cells only depends on cell migration during wound closure. As previously reported, the proliferation of ECs in response to wounding did not start in the first 24 h[12]. Photographs were taken in this study directly after wounding and 24 h after wounding in order to observe the changes of ECs migration (fig. 3A). The percentage of wound closure in ezrin-shRNA transfected cells was significantly lower than that in empty vector transfected cells (47.73%±8.89% *vs*. 71.69%±10.6%, $P<0.05$) (fig. 3B). These results suggested that the capacity of HUVECs migration *in vitro* can be decreased by decreasing the expression level of ezrin.

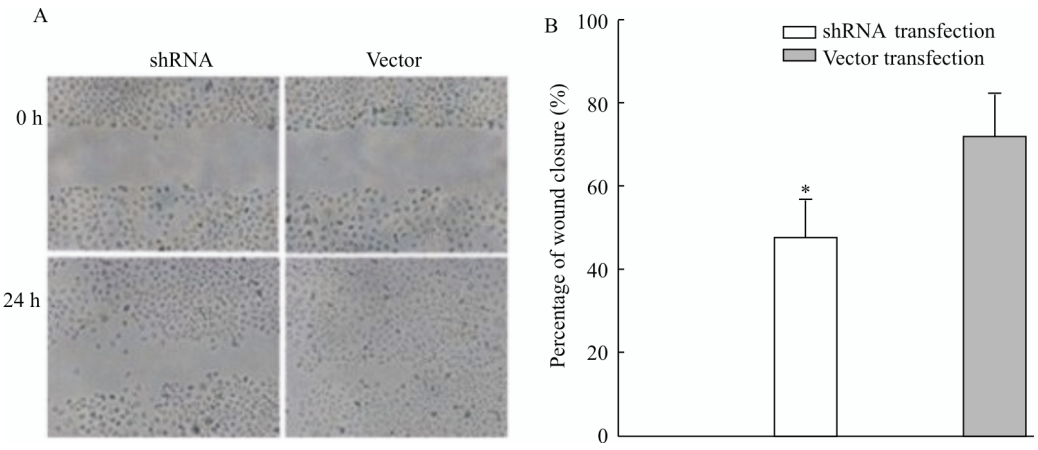

Fig. 3 Effects of ezrin silencing on ECs migration *in vitro*

A: Confluent monolayers of HUVECs were wounded. Photographs were taken directly after wounding (t=0 h) and 24 h after wounding (t=24 h). B: quantification of the endothelial wound repair. ECs migration was quantified 24 h after wounding. Values are $\bar{x}\pm s$ from 6 cultures in 3 independent experiments. The percentage of wound closure in ezrin-shRNA transfected cells was significantly lower than that of empty vector transfectd cells ($^*P$=0.04 *vs*. vector transfection, n=3).

2.4 Effects of Ezrin Suppression on the Formation of Tubular Networks in Three-dimensional Gels

Three-dimensional type Ⅰ collagen provoked HUVECs in cultures to undergo marked shape changes that closely imitated precapillary cord formation. *In vitro* angiogenesis tube formation assay showed that the mean numbers of cordlike structures of 6 randomly chosen microscopic fields (7.3 mm^2 /field) were obtained and compared between groups. As shown in fig. 4A, the results showed that knockdown of Ezrin reduced the organization of ECs into cords (fig. 4B). The number of cordlike structures was 1.83±0.98 and 3.33±1.21 in ezrin-shRNA and empty vector transfected cells, respectively, with difference being significant ($P<0.05$, fig. 4B, n=6). Inhibition of ezrin decreased the capability of EC angiogenesis *in vitro*, suggesting that ezrin as a critical regulator of cytoskeleton mediated the assembly of ECs into precapillary cords within the three-dimensional collagen matrix.

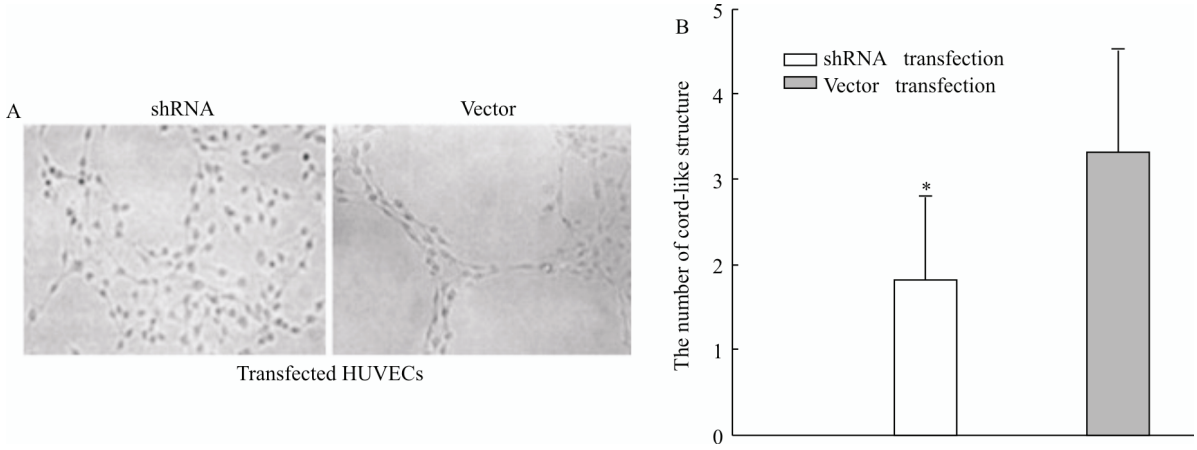

Fig. 4 Effects of ezrin silencing on ECs organization into precapillary cords

A: Confluent ECs monolayers, consisting of equal numbers of cells, were seeded on collagen Ⅰ gels and observed and photographed 4 days later. B: comparison of the number of precapillary cords. The quantification of ECs assembly into cords with morphometry indicated that ezrin silencing reduced cord formation ($^*P$=0.04 *vs*. vector transfection, n=6).

3 DISCUSSION

Although many of the factors (e.g., VEGFs, FGFs, TGF-β) that control the process of angiogenesis are well examined, little is known about the molecular mechanisms by which ECs physically assemble into capillary tube structures in three-dimensional ECM environments. To form three-dimensional capillary tubes, ECs must establish contacts with the ECM that provides signals for their proliferation, migration, and differentiation[16, 17]. During angiogenesis, proliferating ECs are organized to form new three-dimensional capillary networks through a process involving the transition of endothelial precursor cells to spindle-shape morphology in combination with alignment into solid, multicellular, precapillary, cord-like structures[18]. Moreover, these cordlike structures are interconnected to form a polygonal network[19]. Recent studies revealed that ezrin proteins act as linkers between the plasma membrane and the cortical actin cytoskeleton, and play an important role in the formation of microvilli, cell-cell adhesion, maintenance of cell shape, cell motility, and membrane trafficking[20–23]. Thus, the importance of ezrin for these processes is consistent with a possible role in vascular morphogenesis. In the present study, we provided evidence that ezrin is a novel regulator in vascularization.

As shown in this study, we observed a strong reduction in cell motility in HUVECs treated with specific siRNA targeting ezrin. The contractile actomyosin cytoskeleton and its connection to the plasma membrane are critical for control of cell migration. Ezrin protein expression is localized in a specific cell membrane region and is mainly involved in the connections between epithelial cell cytoskeletons and cell membranes[24]. Through membrane surface signaling molecules and some transmembrane signal transduction pathways, ezrin participates in the regulation of cellular survival, adhesion and migration processes. Active ezrin C-terminals connect with the actin cytoskeleton and the N-terminals connect with the cell adhesion molecules such as E-cadherin and CD44[25]. A relevant study also found that through the activation of ras homolog gene family, member A (RhoA) and mitogen-activated protein kinase (MAPK) pathways, ezrin could promote cell adhesion plaque formation, thereby promoting the adhesion function between the ECs and other cells[26]. Recent studies have demonstrated that PIP2 binding and phosphorylation state of ezrin acted in synergy to induce protein activation and thus increased the affinity for filamentous actin[27]. Therefore, we believe that, through participation in the formation of the cell adhesion plaques, cytoskeletal connections and cell surface compound assembly, as well as in other biological functions, ezrin protein mediates and regulates cell adhesion and actin dynamics processes and is involved in the cell migration and angiogenesis processes.

ECs morphogenesis is defined as the process in which ECs assemble into tubes in three-dimensional extracellular matrices. These events require ECs interactions with ECM through integrins, and signaling events involving cytoskeletal elements that control ECs shape and cell-cell interactions that dictate the three-dimensional structure of tubes[18]. How these interactions lead to the ability of ECs to assemble into tubes with a fluid-filled lumen, an abluminal surface in contact with basement membrane matrix, and cell-cell junctional contacts remains unclear. In order for the cell to function well within a multicellular system, the mechanical properties of the plasma membrane need to meet two different requirements: cell shape maintenance and rearrangement[28, 29]. To achieve these goals, ezrin plays key roles in the regulation of the cortical actin cytoskeleton[30–32]. In this study, we showed that the HUVECs with down-expression of ezrin showed malformations, having lower levels of focal adhesion and lamellipodia and filopodia formation, with defined size. The data suggests that ezrin plays a significant role in the maintenance of normal cell morphology. We therefore propose that inhibition of ezrin may result in loss of membrane tension which is normally maintained by ezrin connections between the membrane and the cytoskeleton, which in turn contributes to a loss of cellular morphology control. There was a strong reduction in the number of precapillary cords in HUVECs treated with specific ezrin-targeting siRNA, suggesting that ezrin is a key effector for cord formation.

In summary, this study suggests that ezrin plays an important role in the migration and angiogenesis of ECs *in vitro* by affecting actin organization, indicating the practical importance of regulating vascular morphogenesis by the knockdown of ezrin, which provides a basis for exploring new strategies to control cancers.

Conflict of Interest Statement

The authors declare that there is no conflict of interest with any financial organization or corporation or individual that can inappropriately influence this work.

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(Receive Oct. 13, 2015; revised Dec. 13, 2015)