An experimental study of a modified Dahuang Zhechong pill on the angiogenesis of RF/6A cells in vitro

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OBJECTIVE: To investigate the effects of a modified Dahuang Zhechong Pill (MDZP) on the angiogenesis of rhesus choroid-retina endothelial (RF/6A) cells and its preliminary mechanism.

METHODS: A 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) method was used to assess the effect of a MDZP on RF/6A cell proliferation induced by vascular endothelial growth factor (VEGF). Transwell inserts were used to assess the effect of the MDZP on RF/6A cell migration. Matrigel was used to assess the effect of the MDZP on the tube formation of RF/6A cells. Western blotting and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) were used to detect the protein and mRNA expression, respectively, of VEGF and matrix metalloproteinase-2 (MMP-2) in RF/6A cells treated with the MDZP.

RESULTS: RF/6A cell proliferation induced by VEGF was inhibited by 0.2 mg/mL MDZP. At 0, 12.5, 25 and 50 mg/mL MDZP, the number of cells that migrated through Transwell membranes was 73.33±4.51, 61.33±4.04, 28.67±6.66 and 17.67±4.16, respectively, and the number of tubes formed in Matrigel was 20.33±0.58, 13.33±1.53, 11.00±1.00 and 1.33±0.58, respectively. At 100 and 200 mg/mL MDZP, the protein and mRNA expression of VEGF and MMP-2 were inhibited in RF/6A cells. At 400 mg/mL MDZP, the expression of VEGF mRNA and MMP-2 protein were inhibited in RF/6A cells.

CONCLUSIONS: MDZP inhibits the angiogenesis of RF/6A cells via the suppression of proliferation, migration and tube formation of RF/6A cells. Inhibition of the protein and mRNA expression of VEGF and MMP-2 in RF/6A cells may be an important mechanism.

Key words: Modified dahuang zhechong pill; Rhesus choroid-retina endothelial cell line; Proliferation; Migration; Tube Formation; Angiogenesis; Choroidal neovascularization; Vascular endothelial growth factor; Matrix metalloproteinase-2

INTRODUCTION

Choroidal neovascularization (CNV) can be found in wet age-related macular degeneration (AMD) and other eye diseases. CNV often occurs in the macula, causing repeated bleeding, leakage and scar formation resulting in serious damage to visual function. The mechanism of CNV formation is very complicated. Various
cytokines and signal transduction pathways play a part in the occurrence and development of CNV. Currently, anti-vascular drugs that target endothelial growth factor (VEGF), which are injected into the vitreous cavity, and photodynamic therapy (PDT) are the latest drugs and treatment technology of CNV. However, the occurrence and development of CNV cannot be completely blocked and may relapse and produce various complications. Moreover, these methods and drugs are generally expensive, and broad application for treating the majority of patients in our country is limited. Therefore, investigating safe, effective, inexpensive and easily applied methods and drugs for the treatment of CNV is extremely urgent.

Based on dry blood vacuity taxation treated by the Dahuang Zhechong Pill (DZP) in Golden Chamber (《金匮要略》) and modern medical findings, it is believed that the funduscopy manifestations of fundus macular hemorrhage due to CNV are linked to the internal dry blood and darkish eyes of dry blood vacuity taxation described by Zhang Zhong-jing. Thus, the traditional Chinese medicine (TCM) assumption that the pathomechanism of CNV formation is the dry blood within eyes as the tip and generalized vacuity taxation as the root was proposed. The formula of a Modified Dahuang Zhechong Pill (MDZP) was composed to treat patients with CNV-related diseases including AMD, which achieved a marked improvement in visual function via promoting absorption of hemorrhages and alleviating edema and membrane formation. Therefore, in this study, we observed the effects of MDZPs on proliferation, migration and tube formation, as well as VEGF and matrix metalloproteinase 2 (MMP-2) expression in rhesus choroid-retina endothelial (RF/6A) cell line in vitro. Our aim was to clearly understand the role that MDZPs play in manipulating the angiogenesis of RF/6A cells and explore its preliminary mechanism to provide an experimental basis for clinical application of MDZPs to treat CNV-related diseases such as wet AMD.

**MATERIALS AND METHODS**

**Materials**
The rhesus choroid-retina endothelial cell line RF/6A (Cell Bank of the Chinese Academy of Sciences) was purchased from the Cell Resource Center of the Shanghai Institute of Health Science of the Chinese Academy of Sciences (Shanghai, China).

The TCM compound formula for the MDZP was modified from the classic formula for the Dahuang Zhechong Pill. Chinese medicinal materials (63 g) were soaked in double-distilled water for 60 min, boiled for 30 min and then filtered. The residues were combined with double-distilled water, boiled for 20 min and filtered again. The two parts of the filtrate were mixed and filtrated again, followed by precipitation with 95% ethanol. The ethanol was decompressed and recovered. The water decoction was concentrated to 1 g crude drug/mL. The supernatant was obtained by centrifugation at 3000 rpm followed by filter sterilization through a microporous membrane (0.22 μm) and then stored at −30°C. The supernatant was diluted to the desired concentration with RPMI 1640 medium. Except for Meng Chong (Tabanus Bivittatus) and Qi Cao (dried grub larva), which were provided by Mr. Zhu Jin-er of Shanghai Caitong Detang Pharmaceutical Factory for Chinese Materia Medica (Shanghai, China), raw medicinal materials were purchased from the Pharmacy of the First People’s Hospital Affiliated with Shanghai Jiaotong University (Shanghai, China).

Recombinant human VEGF (V7259) and protamine sulfate (P4020), Sigma-Aldrich Co., (St. Louis, MO, USA), were dissolved in phosphate-buffered saline (PBS) at 10 ng/μL and 1 mg/mL, respectively, and stored at −30°C. Novel tetrazole derivatives MTS (G3580), Promega Corporation (Madison, WI, USA), was purchased from Shanghai Sheng Zhao Biotechnology Co., Ltd.; Transwell chambers (3422), Corning Incorporated (Corning, NY, USA), were purchased from Shanghai Ji Tai Biotechnology Co., Ltd., and Matrigel (354234), BD (Franklin Lakes, NJ USA), was purchased from Genetic Science and Technology Co., Ltd. (Shanghai, China).

Rabbit anti-human VEGF165 affinity purified polyclonal antibody (AB1442), Millipore (CHEMICON), and rabbit anti-MMP-2 (hinge region) polyclonal antibody (AB809), Millipore (CHEMICON), were purchased from Shanghai Ming Rui Bio-Technology Co., Ltd.

A cell and tissue total protein extraction kit (KC-415), Shanghai Kang Cheng Bioengineering Co., Ltd. Shanghai, China); BCA protein assay kit (AR0146), Wuhan Boster Bioengineering Co., Ltd. (Wuhan, China); low-temperature centrifuge, Shanghai An Ting Science and Medical Instrument Factory (Shanghai, China); Microplate Reader, Thermo Fisher Scientific Inc. (Waltham, MA, USA); SYBR Green I RT-PCR Master Mix, TOYOBO Co. Ltd. (Osaka, Japan); RT-kit, Fermentas International Inc. (Burlington Ontario, Canada); SuperScript II reverse transcriptase, Invitrogen Corporation (Grand Island, NY, USA); DU600-UV spectrophotometer, Beckman, Inc. (Brea, CA, USA); quantitative real-time PCR instrument, ABI Prism 7300, Applied Biosystems, Inc. (Foster City, CA, USA).
**MTS Cell Proliferation Assay**
RF/6A cells in a logarithmic growth phase were collected and adjusted to \(8 \times 10^4\) cells/mL with RPMI 1640 supplemented with 10% calf serum and then incubated in 96 well culture plates (100 µL cell suspension per well) at 37°C in a 5% CO2 incubator. After 24 h, cells were washed twice PBS and then incubated for another 24 h in serum-free culture medium. Then, 100 µL various conditional serum-free culture medium was added to wells. After 20 µL MTS was added to each well, cells were incubated in the dark at 37°C for 3 h. The optic absorption (A value) of each well was then measured at 490 nm and the \(A_{490}\) value was analyzed to indicate cell proliferation levels. Eight wells of cells were used for each group. The experiment was repeated at least three times.

**Cell Migration Assay with Transwell Chambers**
RPMI 1640 culture medium was added to a 12 well plate and then to the transwell inserts. The plate was then incubated at 37°C in a 5% CO2 incubator overnight. Transwell inserts contained fresh RPMI 1640 medium supplemented with 10% calf serum (600 µL medium per well), and serum-free culture medium and cells in the inside compartment. RF/6A cell density was adjusted to \(4 \times 10^4\) cells/mL. Cell were incubated at 37°C with 5% CO2 for 24 h in 100 µL various conditional serum-free culture medium. Then, transwell inserts were removed, the liquid inside was absorbed with a dry cotton swab, and cells on the surface of the polycarbonate membrane were gently wiped with a distilled water-soaked cotton swab. Cells were fixed with 4% paraformaldehyde for 20 min and then PBS washed three times. The polycarbonate film was carefully cut from the base of the chamber and mounted on a glass slide. After staining with hematoxylin for 1 min, samples were rinsed several times with tap water and then transparentized with dimethylbenzene for 2–3 min followed by mounting with neutral resin. Cells attached to the lower surface of the polycarbonate membrane were photographed in five randomly chosen visual fields under a microscope. The number of cells that migrated underneath the transwell membrane was counted and averaged with IPP 6.0 image analysis software. Three wells of cells were used for each group. The experiment was repeated at least three times.

**Tube Formation Assay of Endothelial Cells with Matrigel**
Matrigel was thawed at 4°C overnight. Equipment and 96 well plates were placed on ice prior to the procedure. Liquid Matrigel (100 µL) was slowly added to each well followed by carefully shaking for even distribution. All the above processes were carried out on ice. Then, the culture plate was incubated at 37°C with 5% CO2 for 0.5–1 h. RF/6A cells were collected and adjusted to \(1.6 \times 10^4\) cells/mL with RPMI 1640 supplemented with 20% calf serum and then incubated in the 96 well culture plate (50 µL cell suspension and 50 µL conditional serum-free medium per well) at 37°C with 5% CO2 for 24 h. Cells in five randomly chosen visual fields were recorded under a phase contrast microscope after 3, 6, 12 and 24 h. The number of full-formed tubes was counted and averaged\(^3\). Three wells of cells were used for each group. The experiment was repeated at least three times.

**Western Blotting**
RF/6A cells were collected and adjusted to \(10^6\) cells/mL with RPMI 1640 supplemented with 10% calf serum and incubated in culture dishes (4 mL cell suspension per dish) at 37°C with 5% CO2 until attached. Cells were then incubated with serum-free culture medium for 4 h and then serum-free culture medium containing 10 ng/mL VEGF and experimental drugs at various concentrations for 24 h. Four dishes of cells were used for each group. Cells were PBS washed twice, and total protein extraction reagents containing protease inhibitors were added to dishes followed by gentle shaking for 5 min. Cells were transferred to a centrifuge tube and shaken for 15 min in an ice bath, then centrifuged at 12000×g for 15 min to collect the supernatant. Protein concentration was measured according to the manufacturer.

**Quantitative Real-time RT-PCR**
RF/6A cells were collected and adjusted to \(10^6\) cells/mL with RPMI 1640 supplemented with 10% calf serum and then incubated in a 6 well culture plate (2 mL cell suspension per well) at 37°C with 5% CO2 until attached. Cells were then incubated with serum-free culture medium for 4 h and then serum-free culture medium containing 10 ng/mL VEGF and the experimental drug at various concentrations for 24 h. Four wells of cells were used for each group. Total RNA was extracted with Trizol reagent (Invitrogen). RNA concentration was determined by a UV spectrophotometer. RNA quality was assessed by 1% formaldehyde denaturing gel electrophoresis. Reverse transcription was carried out according to the manufacturer’s instructions for Superscript II (Invitrogen Corporation), in which cDNA was obtained and stored at ~20°C. VEGF forward primer: 5’ TTACCTGGCGACTGAGA- AA 3’, reverse primer: 5’ GAGTGCCAGTGAG- GTTTT 3’, product length 145 bp. MMP-2 forward primer: 5’ GTGGATGATGCCTTTGCT 3’, reverse primer: 5’ TCCGTCCTTACCGTCAAA 3’, product length 150 bp. Housekeeping gene \(\beta\)-actin forward primer: 5’ GTGGATGATGCCTTTGCT 3’, reverse primer: 5’ TCCGTCCTTACCGTCAAA 3’, product length 150 bp.
The expression level of each sample was relative to the gene expression level of the drug group was expressed as a multiple with that in the control group, the relative gene expression level in the experimental drug group was relative to that in the control group gene and expressed as \( \frac{Ct\text{ (target gene)}}{Ct\text{ (control group)}} \). The target gene expression level of each sample was relative to the \( \beta\)-actin gene and calculated based on \( 2^{-\Delta\Delta Ct} \). \( \Delta Ct = Ct\text{ (target gene)} - Ct\text{ (\( \beta\)-actin)}. \)

### Statistical Analysis
Measurement data were expressed as the mean ± standard deviation (\( \bar{x} \pm s \)). SPSS 14.0 software was used for statistical analysis. One-way analysis of variance was used for comparisons between groups. Treatment groups were compared to a control group with Dunnett’s tests. A P value less than 0.05 was considered statistically significant.

## RESULTS

### Effects of the MDZP on RF/6A Cell Proliferation Induced by VEGF

MTS experiments showed that compared with that of group 2, i.e. the VEGF-induced RF/6A cell proliferation group, the \( A \) values of group 3, i.e. the protamine positive control group and group 5, i.e. MDZP at the concentration of 0.2 g/mL were significantly reduced with high statistical significance (\( P<0.01 \)) (Table 2, Figure 1), indicating that 12.5, 25 and 50 mg/mL MDZP inhibited RF/6A cell proliferation in a dose-dependent manner.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Concentration</th>
<th>A value (( \bar{x} \pm SD ))</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>1.82±0.10</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>VEGF 10ng/mL</td>
<td>2.10±0.06</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>VEGF Protamine</td>
<td>1.38±0.04</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>VEGF MDZP 0.1g/mL</td>
<td>2.03±0.13</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>VEGF MDZP 0.2g/mL</td>
<td>1.39±0.01</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>VEGF MDZP 0.4g/mL</td>
<td>2.07±0.11</td>
<td>8</td>
</tr>
</tbody>
</table>

Notes: \( ^* P<0.05 \) vs. group 2.

### Effects of the MDZP on RF/6A Cell Migration

Transwell chamber experiments showed that compared with that of group 1, i.e. the negative control group, the number of cells that migrated underneath the transwell membrane of the MDZP group at 12.5 mg/mL was reduced with statistical significance (\( P<0.05 \)), while that of the MDZP group at 25 and 50 mg/mL was reduced with high statistical significance (\( P<0.01 \)) (Table 3, Figure 1). This result suggested that 12.5, 25 and 50 mg/mL MDZP inhibited RF/6A cell migration in a dose-dependent manner.

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug Concentration</th>
<th>Cells (( \bar{x} \pm SD ))</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>73.3±4.51</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>MDZP 12.5 mg/mL</td>
<td>61.3±4.04</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>MDZP 25 mg/mL</td>
<td>28.7±4.66</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>MDZP 50 mg/mL</td>
<td>17.7±4.16</td>
<td>3</td>
</tr>
</tbody>
</table>

Notes: \( ^* P<0.05 \), \( ** P<0.01 \) vs. group 1.

### Effects of the MDZP on the Tube Formation of RF/6A Cells

After 3 h of culture, tube formation of group 1, i.e. the negative control group was observed in Matrigel experiments. At 12 h, tube formation was most evident and did not significantly change thereafter, therefore images at 12 h were used to observe tube formation. Compared with that of group 1, the number of tubes formed in Matrigel by the MDZP group at 12.5, 25, and 50 mg/mL was reduced with high statistical significance (\( P<0.01 \)) (Table 3, Figure 2), indicating that 12.5, 25 and 50 mg/mL MDZP inhibited the tube formation of RF/6A cells in a dose-dependent manner.

### VEGF and MMP-2 Protein Expression

Western blotting showed that compared with that of group 1, i.e. the negative control group, VEGF and MMP-2 protein expression in group 3 – 5, i.e. 100, 200, and 400 mg/mL MDZP, and group 2, i.e. the 200 \( \mu \)g/mL protamine sulfate group, were reduced with high statistical significance (\( P<0.01 \)), but that of VEGF at 400 mg/mL did not significantly reduce (Figure 3). This result indicated that MDZP at the corresponding concentrations inhibited MMP-2 protein expression in RF/6A cells.
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Table 3 Effects of the MDZP on the Tube Formation of RF/6A Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Concentration</th>
<th>Tubes (x ±SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>20.33±0.58</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>MDZP</td>
<td>12.5 mg/ml</td>
<td>13.33±1.53**</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>MDZP</td>
<td>25 mg/ml</td>
<td>11.00±1.00**</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>MDZP</td>
<td>50 mg/ml</td>
<td>1.33±0.58**</td>
<td>3</td>
</tr>
</tbody>
</table>

Notes: ★★P<0.01 vs. group 1.

Figure 4 RNA Denaturing Polyacrylamide Gel Electrophoresis.

Specificity of PCR Products: Specific peaks of melting curves indicated few non-specific PCR products and few primer dimmers. The results of non-specific peaks were removed (Figure 5).

RT-PCR Results: Quantitative real-time RT-PCR showed that compared with that of group 1, i.e. the negative control group, VEGF and MMP-2 mRNA expression in group 3 – 5 i.e. 100, 200 and 400 mg/mL MDZP, and group 2, i.e. the 200 μg/mL protamine sulfate group, were significantly reduced (P<0.05), but that of MMP-2 at 400 mg/mL MDZP was not significantly reduced (Figure 6). This result indicated that MDZP at the corresponding concentrations inhibited VEGF and MMP-2 mRNA expression in RF/6A cells.

VEGF and MMP-2 mRNA Expression
RNA Quality: UV spectrophotometer analysis indicated that the A260/A280 ratios were 1.8 – 2.0, suggesting that RNA purity was adequate. RNA concentrations were 0.2 – 1.2 g/mL. Ribosomal 18s and 28s subunit bands in 1% formaldehyde denaturing gel electrophoresis were clearly visible, indicating high RNA integrity, with no obvious degradation (Figure 4).
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Figure 5 Amplification A and melting B curves (three peaks represent MMP-2, VEGF and β-actin, respectively) of quantitative real-time PCR.

Figure 6 VEGF and MMP-2 mRNA Expression. (Group 1 i.e. the negative control group, group 2 i.e. the protamine control group, group 3–5 i.e. 100, 200 and 400 mg/mL MDZP respectively. *P<0.05 vs. group 1)

DISCUSSION

Angiogenesis is a series of complicated biological processes, which involves vascular dilation, increased vascular permeability and degradation of the surrounding matrix, followed by activation, migration, proliferation, budding growth and tube formation of endothelial cells, with remodeling of neovascularization resulting in vascular nets [1,8]. CNV formation is a process of complex morphological changes involving various elements, which are strictly regulated. It consists of basement membrane degradation, endothelial cell migration and proliferation, and capillary formation. Despite the fact that its formation mechanism has not been fully clarified, injury or composition changes of the Bruch membrane, MMP activation and expression changes of vascular growth and inhibitory factors are important steps in the early stage of CNV.

Various cytokines are involved in the regulation of CNV formation, among which VEGF is presently believed to be the most important. VEGF is believed to be the most powerful factor to promote angiogenesis and is an endothelial cell mitogen, which selectively stimulates proliferation and migration [9] as well as induces anti-apoptotic protein expression in endothelial cells [10], and increases vascular permeability, thereby inducing angiogenesis in vivo. Thus, VEGF is a fundamental factor in regulating angiogenesis and plays a unique role in the regulation of vascular physiology and pathological growth. A slow VEGF increase stimulates the proliferation of choroidal endothelial cells that breakthrough the Bruch membrane and the barrier of the retinal pigment epithelium (RPE), and eventually develops into CNV [11]. VEGF over-expression has been identified as an important factor in the pathological process of CNV-related diseases such as AMD, thus, treatments targeting VEGF such as Lucentis, Avastin, VEGF-trap and small interfering RNA have become a research focus of the prevention and treatment of CNV.

Degradation of the basement membrane and extracellular components caused by an imbalance of the MMP / tissue inhibitor of metalloproteinase (TIMP) system is believed to be the key in CNV formation. MMPs are the most important proteinases involved in degradation of basement membranes and the extracellular matrix, which can degrade almost all components of the extracellular matrix and play a key role in promoting angiogenesis. The current study of the relationship between MMPs and CNV is focused on the gelatinases of the MMPs, namely MMP-2 and MMP-9, which mainly degrade type IV collagen to allow the migration of vascular endothelial cells. In particular, MMP-2 plays an important role in the pathogenesis of CNV. RPE cells are an important source of MMPs in the microenvironment of the outer retina. Cultured human RPE cells secrete MMP-2 in a stationary state. Stimulation with tumor necrosis factor-α, VEGF and fibronectin cause RPE cells to increase MMP-2 secretion and promote CNV formation [12]. MMP expression exists in the CNV membranes of patients with AMD. In the subfoveal vascular membrane of the macula lutea in AMD, MMP-2 is mainly expressed in endothelial cells and dis-
tributed at the formation of new blood vessels\textsuperscript{(35)}.

In this study, various MDZP concentrations affected the angiogenesis of RF/6A cells in vitro. MDZP at 0.2 g/mL inhibited RF/6A cell proliferation induced by VEGF. MDZP at 12.5, 25 and 50 mg/mL inhibited the migration and tube formation of RF/6A cells in a dose-dependent manner. At 100 and 200 mg/mL MDZP, protein and mRNA expression of VEGF and MMP-2 was inhibited in RF/6A cells. At 400 mg/mL MDZP, the expression of VEGF mRNA and MMP-2 protein was inhibited in RF/6A cells.

In conclusion, MDZP inhibits the angiogenesis of RF/6A cells via suppression of proliferation, migration and tube formation of RF/6A cells. The mechanism is likely to be related to the inhibition of mRNA and protein expression of VEGF and MMP-2 in RF/6A cells. DZP has an anti-tumor effect in clinical observation\textsuperscript{(36)} and experimental research\textsuperscript{(37)}, and inhibits angiogenesis in the chick embryo chorioallantoic membrane (CAM)\textsuperscript{(38)}. In our preliminary experiments, MDZP inhibited angiogenesis in the chick CAM. CNV tissue is similar to tumor tissue, in which both are rich in neovascularization. This study provides a preliminary in vitro experimental basis for application of MDZP in the clinical treatment of CNV-related diseases. However, because of its complexity, the relationship between MDZP concentration and its inhibiting effects on proliferation as well as VEGF and MMP-2 expression in RF/6A cells, further study is required. In addition, because of the diversity of MDZP ingredients with varied actions in multiple pathways and targets, its detailed mechanism may not be confined to the inhibition of VEGF and MMP-2 expression, and the mechanism of other aspects still remain to be researched.

Acknowledgment

We thank Dr. Zhao Hui for assistance with experimental methods to analyze cell migration and tube formation.

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