Video Article

A Comprehensive Procedure to Evaluate the *In Vitro* Performance of the Putative Hemangioblastoma Neovascularization Using the Spheroid Sprouting Assay

Ying Wang¹, DanQi Chen¹, MingYu Chen¹, KaiYuan Ji¹, DeXuan Ma¹, LiangFu Zhou¹

¹Department of Neurosurgery, Huashan Hospital, Fudan University

Correspondence to: DeXuan Ma at madexuan03@126.com, LiangFu Zhou at lfzhou@126.com

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Abstract

The inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene plays a crucial role in the development of hemangioblastomas (HBs) within the human central nervous system (CNS). However, both the cytological origin and the evolutionary process of HBs (including neovascularization) remain controversial, and anti-angiogenesis for VHL-HBs, based on classic HB angiogenesis, have produced disappointing results in clinical trials. One major obstacle to the successful clinical translation of anti-vascular treatment is the lack of a thorough understanding of neovascularization in this vascular tumor. In this article, we present a comprehensive procedure to evaluate *in vitro* whether classic tumor angiogenesis exists in HBs, as well as its role in HBs. With this procedure, researchers can accurately understand the complexity of HB neovascularization and identify the function of this common form of angiogenesis in HBs. These protocols can be used to evaluate the most promising anti-vascular therapy for tumors, which has high translational potential either for tumors treatment or for aiding in the optimization of the anti-angiogenic treatment for HBs in future translations. The results highlight the complexity of HB neovascularization and suggest that this common form angiogenesis is only a complementary mechanism in HB neovascularization.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57183/>

Introduction

Hemangioblastomas (HBs) are benign vascular tumors that are found exclusively within the human central nervous system (CNS). They develop in patients with either von Hippel-Lindau (VHL) disease or sporadic lesions. VHL-HBs are difficult to cure through surgical treatment due to the frequent recurrence and multiple lesions that result from this genetic disorder¹. Although the inactivation of the VHL tumor suppressor gene has been considered the root cause of the tumorigenesis of VHL-HBs, the cytological origin (including neovascularization) and evolutionary process of HBs remain largely controversial². Therefore, a better understanding of HB-neovascular biological mechanisms may provide useful insights into the most promising anti-vascular strategies for VHL-HBs.

Recent research has suggested that HB-neovascularization is similar to the embryologic vasculogenesis^{3,4,5}. Classic vascular endothelial growth factor (VEGF)-mediated angiogenesis that originated from the vascular endothelium and that is driven by VHL loss of function resulted in proliferation and neovascular formation, which has been challenged⁶. In 1965, Cancilla and Zimmerman found, using electron microscopy, that HBs originated from the endothelium⁷. Later it was found that stromal cells are derived from vasoformative element⁸. In 1982, Jurco *et al.* found that stromal cells are of endothelial origin⁹. Therefore, we hypothesized that human vascular endothelial cells are the original cells of HB-neovascularization¹⁰. Although it is better to use the primary cultures from HB cells derived from VHL patient surgeries, our previous research indicated that primary cultures from HB are not stable, and cell lines could not be established³. Moreover, the primary cultures in the 3D environment could not identify the cytological origin of HB-neovascularization because they include the progenitors of HB-vascular ingredients^{10,11}. Therefore, as a primitive and classic model of endothelial cells, human vascular endothelial cells (HUVEC) could serve as an alternative cellular model for HBs.

The spheroid sprouting assay is a new model in tissue engineering^{12,13}. In this paper, a 3D collagen-based coculture system *in vitro* using the spheroid sprouting assay was developed, with an overall goal to evaluate whether classic tumor angiogenesis exists in HBs, as well as its role in HBs.

Protocol

This method was performed in accordance with the approved guidelines and regulations of the Research Ethics Committee of Huashan Hospital, Fudan University. Corresponding standard safety measures were followed in each step. For a schematic presentation, please refer to **Figure 1**.

1. Cell Culture and Plasmid Construct

1. Routinely maintain the human umbilical vein endothelial cell (HUVEC) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 unit/mL of penicillin, and 100 µg/mL of streptomycin in a humidified 5% CO₂ incubator at 37 °C.
2. Digest PLKO.1 plasmid with Apal and EcoRI for the synthesis of shRNA fragment. Ensure that the oligonucleotide sequence of VHL shRNA vector is CCGGGATCTGGAAGACCACCCAAATCTCGAGA TTTGGGTGGTCTTCCAGATCTTTTG¹⁴.
3. Then, mix 20 µM system with forward 5 µL of oligo, 5 µL of reverse oligo, 5 µL of 10x NEB buffer and 35 µL of ddH₂O together (The total volume is 50 µL). Heat the mixture at 98 °C for 4 min, and slowly cool down to room temperature in a few hours.
4. Ligate the annealed oligos and PLKO.1 plasmid together with T4 ligase at 4 °C for overnight. 16 h later, add 5 µL of ligation mix into 25 µL competent DH5α cells. Then, screen the successfully ligated plasmids, and verify the inserted fragment by sequencing.

2. Lentivirus Package and Infection

1. In total, mix 10 µg of the PLKO.1-shVHL or PLKO.1-shScramble vector, 7.5 µg of packing plasmid psPAX2, and 2.5 µg of envelope plasmid pMD2.G in 500 µL of the DMEM media without fetal bovine serum (FBS) for 25 min.
2. Add 7 mL DMEM without FBS to the solution prepared above.
3. Culture 293FT cells in the DMEM without FBS in a 10-cm diameter tissue culture dishes. Ensure that the number of 293FT cells is 1×10^7 using a cell counter.
4. Add 1 mL of the solution prepared above to the medium of 293FT cells for transfection.
Note: The 293FT cell line is derived from the 293F cell line and stably express the SV40 large T antigen. Therefore, it is a suitable host for lentiviral production.
5. Replace the culture medium with the DMEM containing 10% FBS after 6 h.
6. Collect the media using a pipette at 48 h after transfection.
Note: The virus exists in the DMEM containing 10% FBS. The dead cells float on the medium. Use 0.45 µm sterile membrane to filter the dead cells and impurities. Generally, there is no need to check multiplicity of infection (MOI). This is to establish a stable cells line. At the last step, all living cells without successful infection will die by puromycin. The shScramble group and HUVEC cells are the control group. Ensure that all the HUVEC cells die with the used puromycin concentration by 72 h. Every well has the same number of cells.
7. Culture the HUVEC cells in the lentiviral media for 72 h. Then, add puromycin to the media of the HUVEC cells at a concentration of 2 µg/mL for 24 h. Use HUVEC cells without any treatment as the control group. Ensure that all control cells die by this time.
Note: The living cells represent a stable cell line of VHL knockdown cells and shScramble cells. The plating density is 5,000/well in 96-well plates.

3. Generation of Endothelial Cell Spheroids

1. Trypsinize the HUVECs cells and resuspend in the DMEM medium with 10% FBS. With a moderate number of cells in a 10-cm culture dish, add 1 mL of trypsin-EDTA.
2. Seed the cells in a 3D round-bottom 96-well plate, at a cell density of 1×10^3 cells/well. Use a well that can accommodate a spheroid of 0.50 µL to suspend the spheroid. Count cells using a cell counter system following the manufacturer's instructions.
3. Incubate the cells at 37 °C and 5% CO₂ continuously for 72 h. Under these conditions, ensure that the suspended cells form the cell spheroids automatically. Replace half of the culture medium after 36 h.

4. In vitro Angiogenesis Assay

1. Thaw the gel solution at 4 °C and dilute it with the reduced serum medium at a dilution ratio of 1:5.
2. Suck the spheroids from the DMEM culture medium with a micropipette. After washing the spheroids with 5 mL of the reduced serum medium, suspend the spheroids gently and cautiously in the diluted gel. Ensure that there are no air bubbles.
3. Embed 300 µL of mixed liquid in a 15-well plate. After incubation at 37 °C and 5% CO₂ for 1 h, add 400 µL the reduced serum medium to each well. Fill sterile water around the wells to generate a humidified environment to hinder evaporation.
4. Thereafter, culture the plate at 37 °C in 5% CO₂ at 100% humidity for 1 h.
5. Carefully aspirate the old medium and replace it by 600 µL of reduced serum medium with 1% endothelial cell growth supplements in each well.
6. After 12 h or 24 h, take images under an inverted light microscope (40*).

5. Data Analysis

1. Upload the images to an online image analysis platform to obtain sprout length data (**Table of Materials**).
2. On the webpage, create an account by email.
3. Then upload the images by clicking the upload button.
4. Download the result by clicking the download button.

Note: The software will generate the processed image and the data. The data contain five parts: cumulative sprout length, mean sprout length, the standard deviation of sprout length, sprout area and spheroid area. In the processed image, each parameter is outlined in a different color to ease identification in the processed image: Red represents sprouts skeleton, yellow the number of sprouts, blue the sprout structure, white sprout end, and orange spheroid.

Representative Results

Original images are taken by inverted light microscope. The typical images of the control group and the VHL group are shown in **Figure 2-A1** and **Figure 2-A2**. The sprouting length of the control group is shorter than that of the VHL group.

After uploading the Images, the online platform provides analysis results directly. The output consists of two parts: the processed image and the corresponding analysis results. The processed images of the control group and the VHL gene silence group are marked with different colors (**Figure 2-A3** and **Figure 2-A4**). The mean sprout length is 65 μm and 125 μm in the control group and the VHL silence group, respectively, and the average cumulative sprout length is 680 μm and 1250 μm in the control group and the VHL silence group, respectively, indicating that sprout length increases by approximately two folds in the VHL silence group, compared with the control group (**Figure 2B**). These results indicate that VHL deficiency promotes the vessel-forming ability of human endothelial cells.

Figure 1. The schema for the spheroid sprouting assay. Steps 1 shows HUVEC cells cultured a dish. In Steps 2, HUVEC cells are moved to 3D round-bottom 96-well plates for 72 h. Steps 3 shows the HUVEC cells form endothelial cell spheroids automatically in the 3D plate. In Steps 4, add cell spheroid to diluted gel. In Steps 5, add the mixture prepared above to a 15-well plate. Steps 6 shows spheroid sprouting in the 15-well plate. Steps 7 show the sprouting image taken under an inverted light microscope. Steps 8 shows the uploaded image and step 9 is the output image from the platform (bar = 100 μm). [Please click here to view a larger version of this figure.](#)

Figure 2. The effect of VHL gene silencing on the angiogenic ability of HUVEC cells in the spheroid sprouting assay. (A) Representative images of spout outgrowth after 12 h in control (A1) and VHL-silenced HUVEC spheroids (A2) (bar = 100 µm). Figures A3 and A4 show the images analyzed by the platform for Figures A1 and A2, respectively. Red represents sprouts skeleton, yellow the number of sprouts, blue the sprout structure, white sprout end, and orange spheroid. (B) Length of the sprouts. Statistical analysis was performed using unpaired Student's t-test. * represent $P < 0.05$. CON, control; HUVEC, human umbilical vein endothelial cell. [Please click here to view a larger version of this figure.](#)

Discussion

Recently, multiple fields of vascular biology research were stimulated by the study of the angiogenic endothelium¹⁵. In this article, we developed an endothelial spheroid sprouting technique as an experimental model to study the vessel formation that originates from the VHL gene's loss of function in manipulated endothelial cells to identify novel candidate molecules of the angiogenic cascade. To the best of our knowledge, this is the first report to examine the angiogenic effects of the endogenous modified endothelial cells.

Tissue (including tumor tissue) neovascularization is a complex process that occurs *via* angiogenic and vasculogenic mechanisms during development, as well as in both physiological and pathological conditions^{12,15,16}. The spheroid sprouting assay is characterized by a complex cascade of events^{17,18}, including the self-aggregation of endothelial cells that are embedded in a 3D cell matrix culture system, which results in sprouting and the reproduction of the 3D network of capillaries¹⁹. This 3D gel-embedded spheroid model has become a widely used system in tissue engineering for studying the vascular formation and its mechanisms due to its ability to provide a better mimicking environment in a suitable matrix. However, the most crucial step of this biological process is the activation of quiescent endothelial cells^{17,20,21,22}. In this procedure, the endothelial cells were activated by the endogenous silencing of the VHL gene, a regulator growth gene of neovessels^{23,24}. Different from the classic endothelium initiation *via* the exogenous induction of the matrix and the corresponding extracellular environment (including exogenous vascular growth factors), this modified method can be extended to numerous applications to unravel endogenous genetic mechanisms. Next, to diminish the effects of the exogenous matrix, the protocol was optimized by diluting the gel, which was a simple step in the experiment. In addition, it took only minutes to upload images to the platform and to obtain analysis results. The platform provides an image analysis service that allows the experimenter to make an objective, comparable, and automated image analysis of sprouting assay images online. Therefore, the protocol overcomes measurement and calculation errors caused by man-made factors.

The mechanistic understanding of the individual steps of the neovascularization cascade provided a rational basis for the development of the anti-vascular treatment that is currently being approved for clinical application in oncology. This paper established a simple and robust spheroid-based assay model to study vessel formation that originates from manipulated endothelial cells with loss of function of the VHL gene to identify novel candidate molecules for the neovascularization cascade, which can be utilized for numerous angiogenesis- and vasculogenesis-related applications. The authors speculate that this *in vitro* model might provide additional information to help evaluate the role of angiogenesis in some solid tumors (e.g., tumor vasculogenic mimicry) and investigate the potential roles of other possible progenitor cells to generate tumor neovessels *in vivo*, particularly in patients with tumors that, as a group, are unresponsive to treatment with anti-angiogenic agents only.

The spheroid sprouting assay has become a widely used method to study angiogenesis and related mechanisms. The assay has an important advantage by providing a better mimic environment than the classical 2D cultures. Recently, 3D co-culture models have been developed to study tumor angiogenesis that mimics the heterogeneity and complexity of angiogenesis within the tumor microenvironment²⁵. In this model, endothelial cells are cultured directly with cancer cells, as well as a stromal component in a 3D environment. In addition, 3D *in vitro* culture systems have been shown to reflect more accurately the *in vivo* response to therapeutic agents than conventional cell culture systems. In addition, this method is used for the differentiation of embryonic stem cells, tissue growth, wound healing, ischemia, and inflammation. Compared with the classic method, our approach is effective and easy to implement. We believe it is a useful tool for the study of angiogenesis *in vitro*, and it opens a new way to understand this process better.

Disclosures

The authors have nothing to disclose.

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