



Ex Vivo Angiogenesis Modeling: A Simple and Cost-Effective Model for Studying Tumor Angiogenesis

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Abstract

Objectives: Endothelial cells (ECs), as the main cell types in the micro- and macro-vascular structure, are crucial to many vital physiological processes occurring in human body among which are both physiologic and pathologic angiogenesis. Due to their great heterogeneity, it is recommended that the ECs of each tissue should be studied separately. Recently, targeting angiogenesis in cancer has become one of the main strategies for cancer management. Accordingly, this study aimed to introduce a simple, cost-effective, and practical model for studying tumor angiogenesis, which can contribute to studies focused on antiangiogenic-based treatments for cancer.

Materials and Methods: In this original study, the explants of both normal and tumor tissues of mouse lung and 4T1 mouse cancer model were cultured separately in a collagen type 1 bed. Sprouting ECs formed a network of vessel-like structures that were photographed as a model for investigating cancerous and normal angiogenesis. These ECs were then isolated from the collagen matrix using collagenase to confirm their endothelial nature.

Results: The results showed that the cells invading the collagen matrix and forming a vessel-like network were of endothelial nature. They were also found to have EC-specific characteristics and express EC-specific markers. These embedded cells may have been treated with the desired drug or further isolated from the collagen matrix and investigated in a genome or protein level.

Conclusions: In sum, this modeling had the potential to become a suitable model for studying the pathological angiogenesis such as tumors in human and animal tissues.

Keywords: Endothelial cells, Neovascularization, Pathologic, Three-dimensional, Tissue culture technique

Introduction

Endothelial cells (ECs), the major components of vasculature in the vertebrates (1), coats the surface of entire blood and lymph circulatory vessels. They are not only the building blocks of vessel walls but are also the dynamic role players in the tissues whilst constituting very small percentage of the cells in tissue (2). Angiogenesis, formation of new blood vessels from pre-existing ones, is also achieved by ECs and induced when proangiogenic factors overcome antiangiogenic ones (3). During this highly supervised and controlled mechanism, degradation of extracellular matrix occurs in order to facilitate the following proliferation, migration, rearrangement of ECs, and formation of a new network of vessels. This process has been detected under both physiological and pathological conditions (4).

New vascularization (neovascularization) in solid tumors is an example of angiogenesis occurring under pathological conditions. In fact, no tumor can grow beyond a limited size without neovascularization (5). When the tumor size exceeds 2 millimeters, the created hypoxia conditions cause the induction of angiogenic factors,

including vascular endothelial growth factor (VEGF), whose concentration gradient towards the adjacent vascular network leads to the induction of angiogenesis in the tumor mass. This vascular network created in the tumor mass provides food supply and removal of waste materials in the tumor, and roots for tumor metastasis (6).

Inhibition of angiogenesis to prevent tumor growth, a theory proposed by Judah Folkman many years ago, is now a solid approach towards cancer treatment (7). Although several antiangiogenic drugs have entered the clinic, there are still shortcomings that could be addressed by further studies in this area. The main drawback halting some anti-angiogenic-based therapies is the inadequate knowledge about tumor ECs and their behavior in the tumor microenvironment (6). Therefore, developing appropriate models capable of closely mimicking the microenvironment of the tumor could enormously contribute to such studies. Although several models have been introduced to carry out such studies, there are still many challenges left to be addressed. The present study, therefore, aimed to introduce a simple and cost-effective model based on explant culture of tumor tissues in a

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Key Messages

- ▶ Endothelial cell lines are not suitable options for anti-angiogenic studies, it is better to investigate angiogenesis in each tissue specifically.
- ▶ Introducing a model that mimics the tumor microenvironment well can be a fundamental step in anti-angiogenic treatments.

3D collagen matrix for angiogenesis studies in order to facilitate developing personalized medicine in the future.

Materials and Methods

Preparation of Collagen Gels

In this study, type 1 collagen was used for both isolating and characterization of the ECs. To extract type I collagen, the rat tail tendons were immersed in a sterile 0.02M acetic acid solution for 48 hours at 4°C on a magnetic stirrer. The extracted solution was sealed in a dialysis bag with cut-off molecular weight of 12 kDa and dialyzed against 0.1X culture medium at pH 4 for another 48 hours at 4°C on a magnetic stirrer. For preparing the model in culture media, 8 volumes of cold collagen solution were mixed with one volume of 10X culture medium and one

volume of sodium bicarbonate (23 mg/mL) in a sterile conical tube (kept on ice to prevent immediate gelation). The mixture was transferred to the culture plate and incubated in a humidified 5% CO₂ incubator at 37°C for 20-30 minutes. After solidification, the complete culture media was added to each well. The ECs were expected to form tubules (vessel-like structures) within the resulting collagen matrix.

Isolation, Culture, and Expansion of ECs From Tumor and Normal Tissues of Mice

To prepare the tumor-bearing mice, a 4T1 mouse mammary tumor cell line was injected subcutaneously in the flank of female inbred BALB/c mice. The vascularized tumor mass was visible and detachable after ten days. Since 4T1 cells are of metastasizing nature, the lung of non-tumor-bearing mice was used for normal ECs (mouse tumor EC: mTEC, mouse lung EC: mLEC). The isolation and culture of ECs from tumor and normal tissues of mice were performed according to the method described and shown in Figure 1 step by step. After anesthetizing nontumor-bearing and tumor-bearing mice

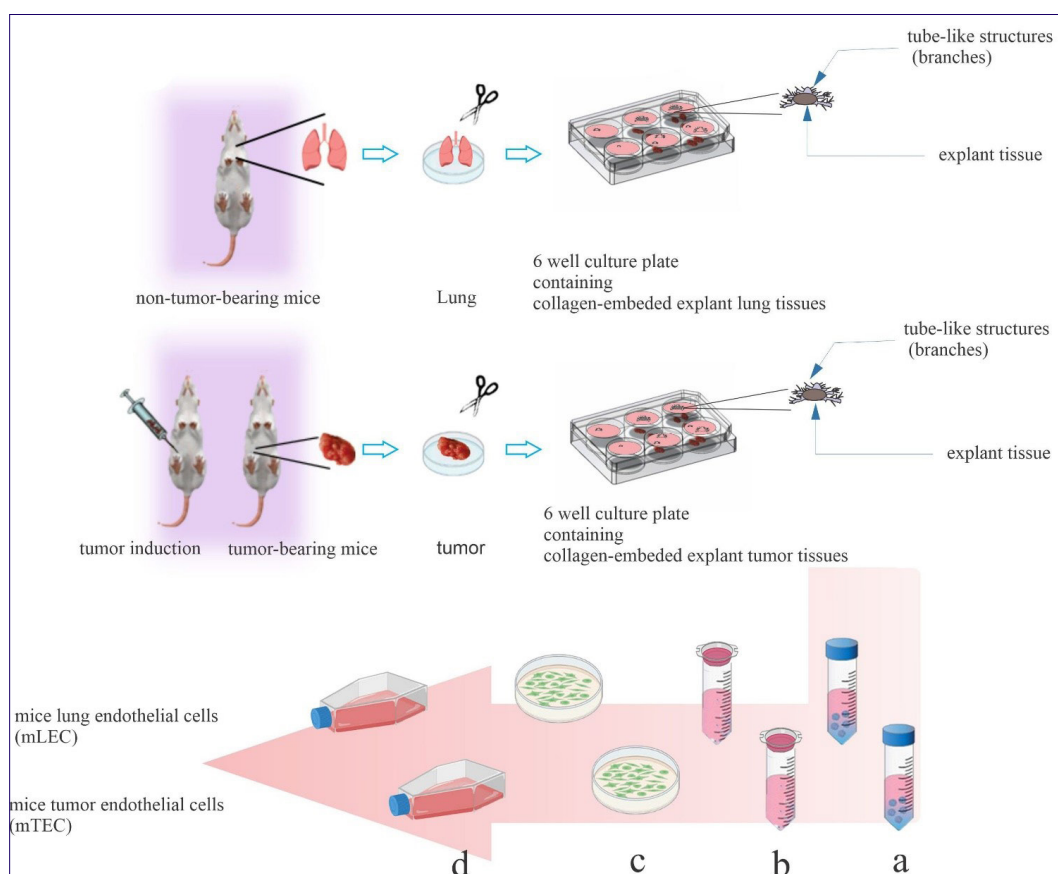


Figure 1. The Schematic Diagram of a Potent Model for Studying Tumor Angiogenesis and Drug Screening. Lung (as normal tissue) and tumor were removed from non-tumor-bearing and tumor-bearing mice, respectively. The dissected lung and tumor tissues were cut into 2-3 mm³ pieces. Cube-like pieces were placed in a well of a 6-well tissue culture plate in order to be embedded in collagen matrix and kept for 30 min in a humidified 5% CO₂ incubator at 37°C to solidify. Complete media was added to each well and incubated without moving for 72 h in a humidified 5% CO₂ incubator at 37°C. To affirm the endothelial nature of migrating cells, isolation from the collagen matrix was performed: (a) collagen matrix-embedded tissue, and collagenase were placed in a sterile conical tube; (b) the suspended cells were transferred from a 70 μm mesh; (c) the mesh transferred cells cultured in a poly-D-lysine coated culture plate; (d) the endothelial cells of both lung and tumor tissue (mLEC: mice lung endothelial cells; mTEC: mice tumor endothelial cells) were subcultured and stored at -80.

with chloroform and sacrificing by decapitation, lung (as normal tissue) and tumor were removed from mentioned mice, respectively.

After rinsing in sterile phosphate-buffered saline (PBS), the dissected tissues were immediately placed in a petri dish containing ice-cold culture medium and then cut into 2-3 mm³ pieces. Afterward, each five to six cube-like pieces were placed in a well of a 6-well tissue culture plate to be embedded in 1.5 mL collagen matrix (with the ratio of 8:1:1 as mentioned earlier) and kept for 30 min in a humidified 5% CO₂ incubator at 37°C to solidify. Then 2 mL of complete media (DMEM/F12 supplemented with 10% FBS, heparin 90 µL/mL, and 6 ng/mL of VEGF) was added to each well and incubated without moving for 72 hours in a humidified 5% CO₂ incubator at 37°C. After 72 hours, the collagen-embedded explant tissues were surveyed for cell migration under an inverted microscope; then, 1 mL of complete media was added to each well. The culture media was exchanged in a ratio of 1:3 every 48 hours for another two or three weeks. ECs were expected to migrate from the collagen embedded tissues (explants) into the collagen matrix and form a vessel-like structure (called branches). Therefore, when the branches were large and expanded enough, the ECs were able to detach from the collagen matrix. So far, this matrix has been known as a potent model for studying tumor angiogenesis and drug screening. To prove the nature of the cells observed to migrate within the matrix, however, performing further cell characterization was required.

To isolate the ECs from the collagen matrix, the following steps were followed: first, each well was emptied of culture media and the collagen matrix was cut into pieces with sterile forceps and pipetting. The mixture was then transferred to a sterile conical tube to be treated with the same volume of collagenase (concentration 2.5 mg/mL) and incubated for 1 hour at 37°C. After centrifugation at 2000 rpm for 3 minutes, the sediment was resuspended in 2 mL of culture media supplemented with 10% FBS, passed through a 70 µm mesh, cultured in a poly-D-lysine coated well of a 6-well plate, and incubated in a humidified 5% CO₂ incubator at 37°C until confluent. The ECs of both origins were used for experiments only from passages 3–8.

Cell Proliferation Assays

The reduction of Tetrazolium salt by living cells during MTT assay was used to examine cell proliferation. For each EC, similar initial cell densities of exponentially growing cells were seeded in a 96-well culture plate with complete media and incubated at 37°C in 5% CO₂. Every 24 hours, a triplicate of cells was examined adopting MTT assay. To perform the test, 3-(4, 5-dimethyl thiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT, Sigma, USA) solution (5 mg/mL in PBS) was added to each well (1:10 ratio) while kept out of light and incubated at 37°C in 5% CO₂ for 4 hours. Then, the supernatants were discarded and the Formazan purple crystals were solubilized using

dimethyl sulfoxide (DMSO) (200 µL/well), and the absorption was measured with a test wavelength of 570 nm and a reference wavelength of 630 nm using an ELISA plate reader. For each cell type, the average absorbance in 24 hours was considered as 100% and was recorded as a reference for following days' cell growth.

Immunocytochemistry

For immunostaining of common EC markers (i.e. CD 31, CD 34, and von Willebrand factor), immunocytochemistry test was carried out. To this end, both mLEC and mTEC were cultured in 24-well culture plates until 70% confluency. Then, the cells were washed three times with washing solution (WS: PBS containing 0.05% Tween) and fixed by adding 4% paraformaldehyde/PBS (room temperature, 20 minutes) followed by rinsing three times with WS. Permeabilizing solution (PBS containing 0.1% Triton X-100) was used for detection of von Willebrand factor. Peroxidase blocking solution was added and incubated at room temperature in a dark place for ten minutes. After washing it with WS, blocking was performed using 1% BSA/PBS at room temperature for 1 h. Then, the blocking solution was removed and the primary antibodies (DAKO) were added, incubated at room temperature for 3 hours, and followed by adding HRP-conjugated secondary antibody (1 hour at room temperature incubation). The cells were washed with WS, and 3,3'-diaminobenzidine (DAB) substrate was added and incubated for five minutes. Then Hematoxylin was added and images were taken using an inverted microscope. It is worth mentioning that washing steps were performed in triplicate and lasted 5 minutes.

Three-Dimensional Tube Formation Assay

Endothelial (mTEC and mLEC), 4T1 (as negative control), and human umbilical vein ECs (HUVECs) (as positive control) cells were cultured in DMEM/F12 with 10% fetal bovine serum (FBS). The 3D angiogenesis assay was performed thoroughly as described in previous studies (8). Accordingly, cells were suspended in DMEM/F12 supplemented with 10% FBS and mixed with the pre-prepared cytodex-3 microcarrier beads in cryotubes gently flipping every 20 minutes for 4 hours at 37°C in 5% CO₂. The mixtures were then transferred to a 24-well tissue culture plate and incubated with additional complete culture media for another 12–16 hours under the same culture conditions. This time allows maximum surface attachment of cells to the microcarrier beads. Afterwards, the cell-incorporated beads were mixed with 500 µL collagen solution (rat tail extracted type I collagen, 10X DMEM/F12, and 23 mg/mL NaHCO₃ with a ratio of 8:1:1, respectively) and distributed in a 24-well plate. After the collagen was solidified in the incubator for 30 minutes at 37°C in 5% CO₂, 500 µL DMEM/F12 with 10% FBS was added to each well. Sprout formation was investigated using an inverted microscope 24, 48, and 72 hours later, and the images were captured.

Results

Formation of Vessel-Like Structure in Collagen Matrix as a Model of Angiogenesis

The EC branches from type 1 collagen-embedded tissues cultured in complete media containing VEGF and heparin were visible after 72-96 hours. Upon reaching an appropriate density of branches forming a vascular network (Figure 2), the embedded cells could be treated with the desired drug, or be further isolated from the collagen matrix and investigated at a genome or protein level.

In order to validate the endothelial nature, the cells had to be isolated from the collagen matrix using collagenase. Since the growth of tumor tissue branches was much faster than that of normal tissues, isolation for tumor tissues was performed after three weeks but that for normal tissues was carried out after four weeks. However, no difference was observed in the doubling time of isolated cells when cultured in flasks (Figure 3h).

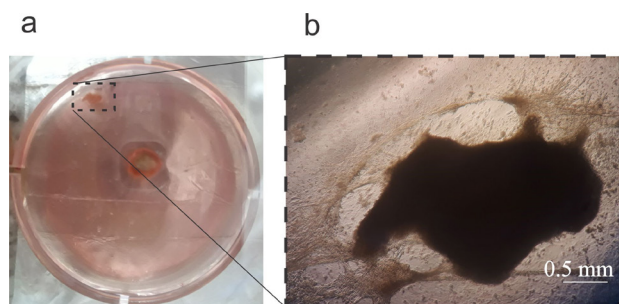


Figure 2. Ex-plant Culture of Mice Tissues in the Collagen Matrix. (a) A well of 6-well culture plate containing mice tissues embedded in collagen (macroscopic view). (b) microscopic view of it.

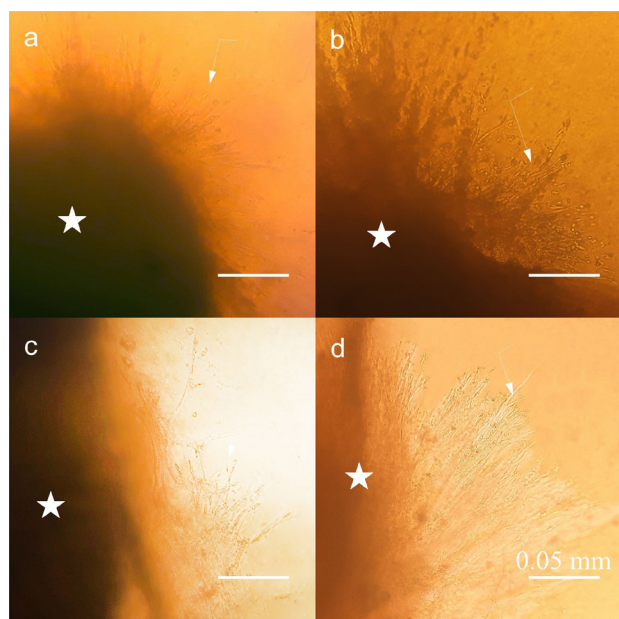


Figure 3. Vessel-like Structure of Endothelial Cells in Collagen Matrix. These structures branched from: 1) Tumor explant culture one (a) and three weeks (b) after culture. 2) Lung explant culture one (c) and four (d) weeks after culture. Stars indicate tissues and arrows indicate branches.

Validation of Endothelial Cells

Cells isolated from collagen were first cultured in a 6-well culture plate coated with poly-D-lysine until suitable confluency favorable for passage was achieved in a 25T flask (Figure 4a and 4d). The cells were fully elongated and spindle-shaped, as expected for ECs morphology. The cells even formed a lumen at the junction in the culture plate.

Lumen and tubule formation was observed in both ECs seeded on collagen (2D culture, Figure 4b and 4e), while 4T1 cells showed neither of such features (not shown).

Angiogenesis potential of ECs was assessed using an *in vitro* 3D culture of collagen-cytodex model. Although this potential in mLEC was greater than mTEC, both EC types, except for 4T1, were able to form tubes in the collagen matrix (Figure 4c, 4f).

Isolated ECs also expressed EC-specific markers. The expression of CD31 and CD34 and von Willebrand factor were examined at a protein level by immunocytochemistry. As shown in Figure 5, both mTEC and mLEC expressed the above-mentioned markers.

Discussion

In the present study, implantation of tissue fragments in the collagen matrix was introduced as an advisable model for the specific study of angiogenesis in each tissue. According to this model, any tissue in which the angiogenesis is under investigation could be simply explanted in a collagen matrix, and the vascular network branched and formed in the collagen matrix is potentially an appropriate tool for the particular study of the same tissue. In this way, the heterogeneity issue of ECs as well as the difference between normal and cancerous angiogenesis are overcome. By using a small piece of tissue, moreover, the required amount of the target ECs can be amplified for a specific purpose. The latter can be a good advantage for its application in personalized medicine.

It has been shown that ECs are very heterogeneous whose characteristics vary based on the tissue they reside in. Interestingly enough, even within one tissue, microvascular ECs vary from those of macrovascular origin (9). Therefore, more specifically targeted studies are required to explore ECs of various tissues separately. Furthermore, it has been repeatedly shown that tumor ECs, as a substantial role player in angiogenesis, are different from normal ECs (10). They exhibit cytogenetic abnormalities and acquired drug resistance (6). As angiogenesis is a hallmark of solid tumors, its inhibition is considered a potent strategy for cancer management (7,10). Hence, several anti-angiogenic drugs (e.g., VEGF pathway inhibitors) have long been introduced to the clinic. However, these drugs have failed to always deliver the expected results since patients have gradually developed resistance to them. The ineffectiveness or partial effectiveness of anti-angiogenic therapies could be explained by the lack of thorough knowledge of these

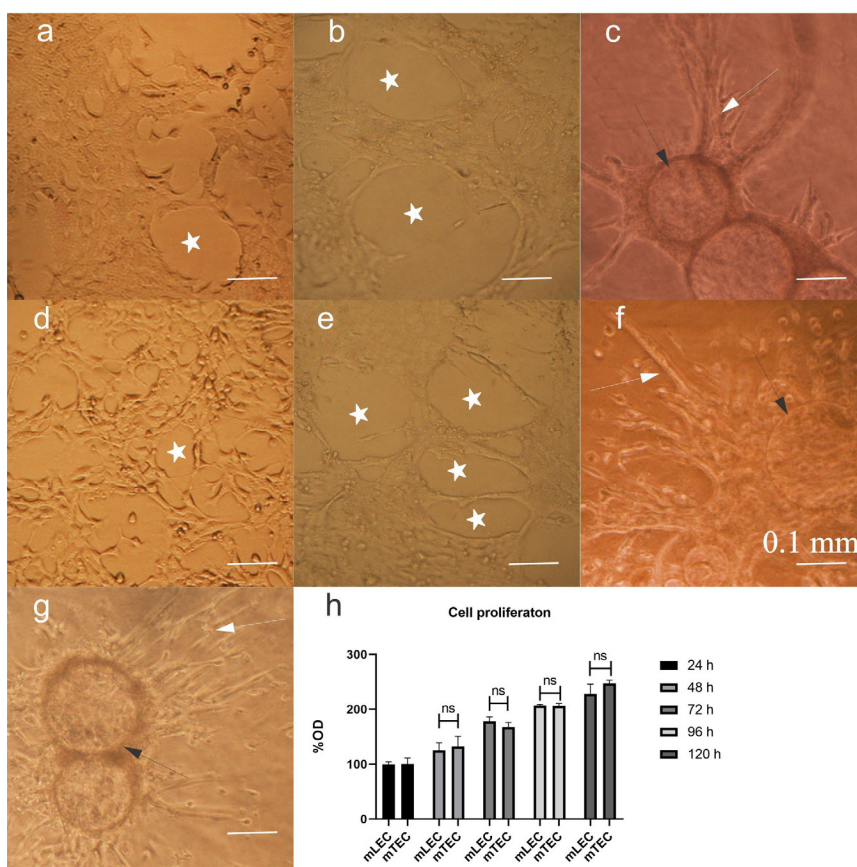


Figure 4. Isolated Mice Endothelial Cells (mTEC and mLEC) in Culture Conditions. (a, b, c) mTEC in a flask, 2D and 3D culture, respectively. (d, e, f) mLEC in a flask, 2D and 3D culture, respectively. (g) 3D culture of HUVEC cells as a positive control. The stars indicate the lumen. Black and white arrows indicate the cytodex and branches, respectively.

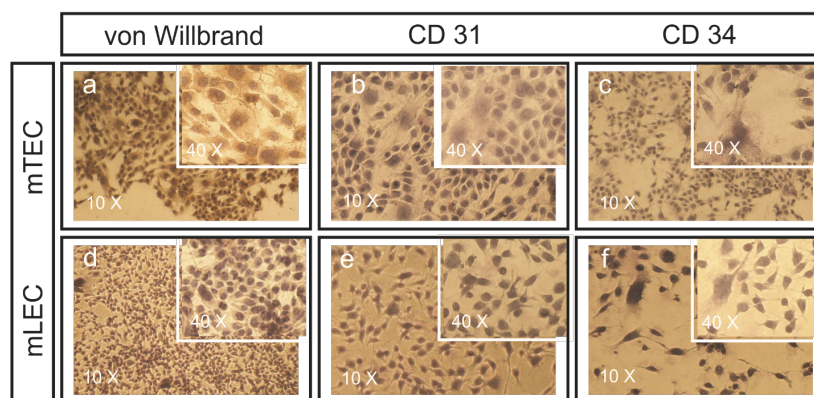


Figure 5. Immunocytochemistry Staining of Mice Endothelial Cells.

cells. Therefore, it is immensely helpful to study ECs of the intended tumor tissue in order to find the most effective angiogenesis inhibition method for approaching the tumor. Therefore, developing applicable models to study the angiogenesis of target tissues could be of great value.

In general, the methods used to study angiogenesis could be divided into three categories of *in vitro*, *ex vivo*, and *in vivo*, each of which has advantages and disadvantages (3). For *in vitro* studies, researchers usually use either EC lines or primary ECs isolated from the target

tissue to study angiogenesis (11). Cell lines, on the other hand, may have different characteristics and behavior from the target tissue ECs. For example, HUVEC, a widely-used cell line in angiogenic studies, is isolated from macrovascular and venous vessels and shows different characteristics from microvascular and arterial ECs (11). Moreover, ECs not isolated from tumor tissue fail to properly reflect the behavior of tumor ECs (6). In addition, cells residing in the tumor microenvironment are exposed to very different factors than those cultured

in flasks. In the tumor microenvironment, the ECs are affected by both the structure of three-dimensional matrix and other cells, which 2D-monolayer culture cannot completely provide (10). Although these shortcomings in cell culture have been partially offset by 3D-cultures as well as co-culture methods with other cells (12), more advanced models are still needed to be introduced into angiogenic studies. *In vivo* studies in animals are also costly and time consuming and, more importantly, require a large number of animals. Therefore, an extra step of initial screening seems necessary before conducting such studies. Given the above discussion, using *ex vivo* studies to investigate tumor angiogenesis is highly recommended and beneficial, specifically for screening of newly developed drugs for anti-angiogenic therapy purposes (3). *Ex vivo* could be defined as organs or parts of organs cultured *in vitro* bridging the *in vitro* to the *in vivo* studies (3,13). Elimination of confounders in *in vivo* studies and simplification of monitoring the results are among the advantages of *ex vivo* method (14).

Although this type of modeling has been introduced and applied in various studies on angiogenesis, it has not yet been used to study tumor angiogenesis. Takahashi et al used *ex-plantation* of spleen, liver, kidney, brain, and muscle tissues in collagen as a new approach for studying ECs of different tissues (15). It is worth mentioning that tissue culture in the collagen matrix has already been used to isolate ECs from the aorta (16).

Nowadays, isolation of ECs from macrovascular vessels by explanting a piece of vessel in collagen/matrigel is a common method (17,18). Although Park et al have cultured tumor tissue explants in matrigel (19), this method is still not quite common.

Since the present study aimed to study tumor angiogenesis, tumor endothelial and normal endothelial were prepared from an animal model of breast cancer and mouse lung tissue, respectively. As expected, the tumor tissue explant formed branches independent of growth factors while the normal tissue relied on them being added to the cell culture medium (data not shown). This is due to the presence of growth and proangiogenic factors in the tumor microenvironment (6). To achieve consistency in culture conditions, however, both tissues were provided with specific endothelial growth factors including VEGF and heparin. As is known, heparin inhibits the growth of smooth muscle cells undesirable to our setting (20) and VEGF is the most important factor in angiogenesis signaling (6). Since this model had been introduced for studying the angiogenesis, ECs branched in the collagen matrix were isolated in order to be characterized immunocytochemically. Although branching and growth of mTEC were greater under explant condition, measuring the doubling time of flask cultures showed no difference in proliferation rate of two cell types *in vitro* (Figure 4h). This clearly highlighted the substantial role of the factors and cells in the tumor microenvironment facilitating

and boosting the sprouting process of mTEC. Although similar growth patterns have been documented in other studies (19), further studies on animals and human biopsy tissues could be designed to support this claim.

EC validations were done on three levels. Morphologically, both isolated cells showed a spindle-like appearance similar to morphology of ECs from arterial sources, validating their endothelial nature on the morphological level. HUVECs, on the other hand, exhibited a cobblestone morphology and were isolated from venous vessels which undergo lower shear stress than arterial vessel cells (21). The expression of endothelial-specific markers was investigated by adopting immunocytochemistry on the protein level. The von Willebrand factor is one of the main and classic endothelial markers whose expression was confirmed in both cell types using immunocytochemistry. Cluster of differentiation 31 (CD 31) or platelet EC adhesion molecule is a transmembrane adhesion protein highly expressed on ECs. For characterization of ECs, the expression of this marker was checked through immunocytochemical staining using specific antibodies against CD 31. Results showed that both cell types expressed this marker, affirming their endothelial nature on the protein level as well. CD34 is another important marker of endothelial progenitor cells whose expression was also confirmed using immunocytochemistry. This marker, among seven other specific genes, has also been introduced as a specific marker of tip cells, the leading cells of a sprout (14,15). Finally, the ability of tube formation in a collagen/matrigel bed is another ECs characterization method on a functional level (21). In this study, both ECs also showed this ability; however, it was superior in mLEC than in mTEC. This observation was reasonable due to the weak connections between tumor ECs.

Although the results of validation tests confirmed the endothelial nature of (mTEC and mLEC) cells and the accuracy of this model, further investigation was found necessary for quantification. Selection of an animal model for this research was based on the simplicity of inducing cancer model, genetic manipulation capacity, and the elimination of genetic differences by using inbred mice. Adopting this method, the desired study was carried out by using only two mice: one as tumor-bearing model and the other as non-tumor bearing (normal) model. Therefore, the '3Rs' principles (i.e., replacement, reduction, and refinement) of animals in product testing and scientific research were met perfectly (20). Interestingly, it was argued that this model may have been also used to examine the human tissues and, after proper confirmation of the model, applied in personalized medicine (22).

Furthermore, this method may have had the advantage of isolating and expanding ECs from various tissues, both animal- and human-originated. It may have effectively and economically contributed to viable researches on the EC biology. For example, it may have been used to study the endothelial dysfunction in many diseases, such as

diabetes, atherosclerosis, hypertension, lung injury and COVID-19, which was the world's largest problem at the time of this study (4,20).

It may have been also employed to isolate ECs from different tissues (normal and pathological) without using multi-step procedures and sophisticated instruments. Most methods used for primary microvascular EC culture are based on enzymatic digestion and cell selection through specific and expensive antibodies, and require special equipment such as flow cytometers (FACS method) (23,24). On the other hand, the selection of these cells based on one type of endothelial marker may lead to the isolation of only one subset from the heterogeneous population of ECs in each tissue. This, in turn, prevents the study to reflect the complete characteristics of the ECs within the given tissue (25). In addition, some of these techniques, such as magnetic-activated cell sorting, may adversely affect cell function as well (20). Matrigel has been sometimes used instead of collagen in similar studies, which not only costs a lot of money but also suffers from some drawbacks, including inducing angiogenesis on its own (18). In this method, therefore, ECs undergo fewer unwanted changes and are closer to normal conditions.

In sum, a simple, time- and cost-effective, and practical model was introduced in our study to investigate ECs from any target tissue (i.e., tumor EC in the present study). However, it was recommended that further studies should be conducted in order to develop a stable and quantitative method for studying ECs, specifically in personalized medicine.

Authors' Contribution

Conceptualization: Azam Rahimpour, Kamran Mansouri, Parisa Mohammadi.

Methodology: Parisa Mohammadi, Kamran Mansouri.

Validation: Kamran Mansouri.

Investigation: Parisa Mohammadi, Farnaz Ahmadi.

Data Curation: Parisa Mohammadi, Farnaz Ahmadi.

Visualization: Parisa Mohammadi.

Supervision: Kamran Mansouri.

Funding Acquisition: Azam Rahimpour, Kamran Mansouri.

Writing—Original Draft Preparation: Parisa Mohammadi.

Writing—Review and Editing: Azam Rahimpour, Kamran Mansouri.

Conflict of Interests

Authors have no conflict of interest.

Ethical Issues

The study procedure was approved by the Research Ethics Committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1399.762).

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