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Fibroblast-Derived 3D Matrix System Applicable to Endothelial Tube Formation Assay

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TITLE:

Fibroblast-Derived 3D Matrix System Applicable to Endothelial Tube Formation Assay

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KEYWORDS:

Tumor Microenvironment, Three-dimensional (3D) culture, Extracellular Matrix, Fibroblasts, Collagen, Neoangiogenesis.

SUMMARY:

The aim of this method is to obtain fibroblast-derived 3D matrices as a natural scaffold for subsequent cellular assays. Fibroblasts are seeded in a pre-treated culture plate and stimulated with ascorbic acid for matrix generation. Matrices are decellularized and blocked to culture relevant cells (e.g., endothelial cells).

ABSTRACT:

The extracellular matrix (ECM) is a three-dimensional scaffold that acts as the main support for cells in tissues. Besides its structural function, the ECM also participates in cell migration, proliferation, and differentiation. Fibroblasts are the main type of cells modifying ECM fiber arrangement and production. In cancer, CAFs (cancer associated fibroblasts) are in permanent

activation status, participating in ECM remodeling, facilitating tumor cell migration, and stimulating tumor-associated angiogenesis, among other pro-tumorigenic roles. The objective of this method is to create a three-dimensional matrix with a fiber composition that is similar to in vivo matrices, using immortalized fibroblasts or human primary CAFs. Fibroblasts are cultured in pre-treated cell culture plates and grown under ascorbic acid stimulation. Then, fibroblasts are removed and matrices are blocked for further cell seeding. In this ECM model, fibroblasts can be activated or modified to generate different kinds of matrix, whose effects can be studied in cell culture. 3D matrices are also shaped by cell signals, like degradation or cross-linking enzymes that might modify fiber distribution. In this context, angiogenesis can be studied, along with other cell types such as epithelial tumor cells.

INTRODUCTION:

The extracellular matrix (ECM) is a dynamic structure present in all types of tissue. It consists of proteins and polysaccharides that create a net of fibers crucial for cell adhesion, migration, and communication[1]. ECM composition varies depending on the tissue. While type-I collagen is the most prevalent structural protein, collagen types II, III, V and XI can also be found in various tissues[2]. Fibronectin generated by fibroblasts is needed for cell adhesion[2]. Moreover, there are other structural molecules like elastin, laminin and surface receptors called integrins that mediate fiber assembly and are specific to the different ECM tissues[2]. The ECM plays an important role as a cell scaffold and can also be involved in both physiological and pathological processes[1]. Abnormalities in the ECM are observed in pathologies such as cancer, which alters ECM composition and/or its organization. In tumors, the ECM represents the non-cellular component of the tumor microenvironment (TME), a complex milieu of cell components such as fibroblasts, immune cells, endothelial cells, pericytes and a variety of soluble factors. It is known that the TME promotes cancer progression and metastasis; cancer-associated fibroblasts, as a predominant cell type in the tumor stroma, take part in this process[3]. Unlike normal fibroblasts, CAFs are in permanent activation, showing increased secretion of ECM proteins and growth factors (e.g., Transforming Growth Factor-β, TGF-β), as well as a higher expression of some markers, such as α-smooth muscle actin (α-SMA) and fibroblast activation protein (FAP)[4]. However, CAFs are a heterogeneous cell population, showing different levels of activation or marker expression[5]. It can be assumed then that the composition and structure of fibroblast-derived matrices will depend on fibroblast status and characteristics.

In this context, the goal of this methodology is to establish an appropriate in vitro model for ECM generation by fibroblasts equivalent to the in vivo ECM setting. We propose this approach as an in vitro translational methodology for further studies of tumor cell functions, like chemoresistance or migration, mediated by ECM. As our group has published elsewhere, CAFs can be obtained from fresh tissue samples, but it has to be noted that the CAFs' survival in culture is limited and their cell passage number is reduced[6]. In addition, CAF primary cultures established from patients´ samples can be used for matrix generation. Manipulation of gene expression in fibroblasts is also an interesting way to produce varied in vitro matrices to assess the possible effects on matrix composition, fiber orientation, etc. Along these lines, our group has recently reported the role of Snail-expressing fibroblasts in the composition and fiber orientation of various derived matrices[7].

Furthermore, CAFs and ECM are involved in the vascular system, both in vessel generation and as part of the vase outer layer[8]. ECM remodeling induces angiogenesis; matrix metalloproteinases (MMPs) seem to be the most important enzyme type contributing to this process[9,10]. The tissue vascularization of primary cells that generate the ECMs, ECM macromolecules, residual growth factors included in the ECM, matrix elasticity, and matrix thickness are described as factors involved in endothelial cell activation[11]. In tumors, hypoxia increases ECM stiffness and endothelial sprout generation[12]. Moreover, CAFs secrete vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) that stimulate angiogenesis in tumor stroma[13]. In this field, in vitro matrix generation could be used to study angiogenesis processes or MMP action under different experimental conditions. Thus, the in vitro reproduction of the most analogous in vivo matrix could be a valuable tool to investigate ECM's role in angiogenesis or micro-environmental cell interactions.

The stimulation of cultured fibroblasts with ascorbic acid to enhance matrix deposition and generate an ECM is an accepted way of producing analogous in vivo matrices. Immortalized fibroblast cell lines are easily cultured and are activated by diverse growth factors, like PDGF-BB, Tumor Necrosis Factor-α (TNF-α) or TGF-β[14]. Within the TME, CAFs synthesize type-I collagen and fibronectin as the main components of ECM[4]. Similarly, these components are found as major components of in vitro-generated fibroblast-derived matrices (**Figure 1**).

There are different in vitro methodologies to simulate in vivo ECM. The use of coated culture dishes with mixtures of ECM fibers was extended in past years, but this 2D approach needed improvement to 3D structures, such as cross-linked gels (e.g., Matrigel)[1]. The Matrigel-like setup has become the standard method for simulating a 3D matrix. Fibrin is also an alternative when generating matrices but fails in terms of strength and durability of the ECM[1]. Collagen used in combination with other ECM components amends some of the abovementioned issues. However, these collagen gels form a strong network with fibers that can be oriented, but are highly heterogeneous, which can be a problem in experiment repetitions[1]. Nevertheless, it must be assumed that, depending on the objective of the experiments, the use of Matrigel or other hydrogels is more appropriate (e.g., in matrix contraction studies in which gel contraction can be easily detected).

The potential immunogenicity of the generated matrices could be an issue in experiments with some cell types. Therefore, to reduce the possibility of immune responses due to ECM-generating cells when using our method, matrices are decellularized and washed, although cell fragment removal could not be total[15]. The ideal ECM needs to be compatible with cell culture and able to communicate and react to cell signals. Our procedure allows the introduction of changes without difficulty during ECM production (e.g., adding fibroblast-stimulating growth factors).

PROTOCOL:

Human tissue samples were obtained with the approval of the Research Ethics Board of the

Hospital Ramón y Cajal, Madrid.

1. Preparation of solutions

1.1. Prepare 0.2% gelatin solution: add 1 g of gelatin to 500 mL of PBS. Autoclave the solution and keep at 4 °C. Filter with a 0.22 µm filter before use.

1.2. Prepare 1% glutaraldehyde: Add 1 mL of 25% glutaraldehyde stock solution to 24 mL of PBS. Filter with a 0.22 µm filter before use.

1.3. Prepare 1 M ethanolamine: prepare ethanolamine solution with sterile H_2O. Filter with a 0.22 µm filter before use.

NOTE: As ethanolamine is provided with a security cap, a needle and syringe will be needed.

1.4. Prepare ascorbic acid: add 0.1 mL of 50 mg/mL stock solution ascorbic acid (light-sensitive) to 100 mL of medium.

1.5. Prepare lysis buffer: prepare PBS 0.5% Triton 100X with 20 nM of NH_4OH. Add NH_4OH right before use.

1.6. Prepare PBS Pen/Strep: dilute Pen/Strep stock solution to 100 U/mL Pen and 100 µg/mL Strep.

1.7. Prepare 10% DMEM: Add 10% FBS to 500 mL of DMEM. Supplement with 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mg/mL Normocin and 0.25 µg/mL amphotericin B.

1.8. Prepare 2% BSA heat denatured: add 2 g of BSA to 100 mL of sterile water. Warm in boiling water for 7 min.

1.9. Prepare FBS with antibiotics: supplement FBS with 200 U/mL penicillin, 200 µg/mL streptomycin, 100 µg/mL gentamicin and 2.5 g/mL amphotericin B.

2. Cell culture preparation

2.1. Immortalized fibroblasts cell line

2.1.1. Culture recombinant telomerase transfected immortalized human foreskin fibroblasts (BJ-hTERT, ATCC CRL-4001) in 10% DMEM in FBS and maintain at 37 °C and 5% CO_2.

2.2. Endothelial cells

2.2.1. Culture human umbilical vein endothelial cells (HUVECs, ATCC PCS-100-013) in EBM-2 medium containing 2% FBS and maintain at 37 °C and 5% CO_2.

2.3. Fibroblast primary culture

NOTE: For CAF establishment and culture, the protocol previously published by our group was followed[6].

2.3.1. Briefly, cut tissue samples into small pieces of approximately 2-3 mm^3 and seed in FBS with high concentration of antibiotics.

2.3.2. When the first fibroblasts appeared, replace medium with FBM medium for cell maintenance.

NOTE: Depending on tissue origin, cell cultures can be contaminated easily. Wash samples in PBS supplemented with antibiotics, with highly contaminated tissue (e.g., colon[6]) and shake for 30-45 min.

3. Fibroblast-derived 3D matrices (Adapted from Castelló-Cros and Cukierman[16])

3.1. Add 2 mL of 0.2% gelatin solution to each well of a 6-well plate and incubate for 1 h at 37 °C or overnight at 4 °C.

3.2. Aspirate gelatin and wash it in 2 mL of PBS.

3.3. Add 2 mL of 1% glutaraldehyde and incubate for 30 min at RT. Glutaraldehyde will cross-link gelatin.

3.4. Aspirate glutaraldehyde and wash wells in 2 mL of PBS for 5 min. Repeat 3 times.

3.5. Add 2 mL of 1 M ethanolamine and incubate for 30 min at RT. Ethanolamine will act to block the remaining glutaraldehyde.

3.6. Aspirate ethanolamine and wash wells in 2 mL of PBS for 5 min. Repeat 3 times.

3.7. Add 1 mL of 10% DMEM. If the medium immediately turns pink, remove the medium, wash in 2 mL of PBS and add again 1 mL of 10% DMEM.

3.8. Seed 1 mL of fibroblast suspension, with 5 x 10^5 cells in each well. Total volume in each well will be 2 mL.

3.9. Culture cells until 100% confluence is reached. Then remove the medium and replace with 10% DMEM with 50 µg/mL ascorbic acid and additional treatment if used.

3.10. Replace medium with fresh 10% DMEM and 50 µg/mL ascorbic acid every 2 days for 6 days.

NOTE: If additional treatment is used (e.g., PDGF-BB, TGF-β and/or other grow factors), add them the same days as ascorbic acid treatment is added.

3.11. Two days after the last ascorbic acid treatment, remove the medium and wash in 2 mL of PBS.

3.12. Slowly add 1 mL of lysis buffer, pre-heated at 37 °C, to each well. Incubate for 5-10 min at RT until fibroblasts are lysed (observable under the microscope).

3.13. Carefully and without removing lysis buffer, add 2 mL of PBS. Then aspirate approximately 2.5 mL of PBS. Repeat twice for a total of three washes.

3.14. Eventually, remove 2.5 mL of PBS and add 2 mL of PBS with Pen/Strep (100 U/mL and 100 µg/mL, respectively). Seal with film and keep at 4 °C for up to 3 months.

NOTE: Depending on the experiment, long-term storage of the generated matrices may affect results. We recommend using matrices as soon as possible, and even more so if the experiment involves cell seeding, due to possible protein degradation. If matrices are used for structural assays, such as collagen observation, matrices can be fixed and stored for longer periods. This protocol is indicated for a 6-well culture plate. Other plates can be used, but reactive volumes and cell suspension concentration need to be recalculated according to well area.

4. Tube formation assay

4.1. Grow HUVEC cells in EBM-2 2% FBS until maximum confluence.

4.2. Replace medium with EBM-2 without FBS for 8 h.

4.3. Prepare matrices before seeding cells.

4.3.1. Remove matrices from the refrigerator and place for 1 h at RT.

4.3.2. Block matrices by adding 2 mL of heat-denatured 2% BSA. Incubate 1 h at 37 °C.

4.3.3. Aspirate BSA and wash in 2 mL of PBS.

4.4. Seed 2 x 10^5 HUVEC cells in FBS-depleted medium on each well of a 6-well plate previously coated with 3D fibroblast-derived matrices.

4.5. Incubate endothelial cells at 37 °C for 16 h.

4.6. Examine tube-like structure formation under a standard bright field microscope at 20-40x magnification.

REPRESENTATIVE RESULTS:

PDGF-BB stimulated fibroblasts create a thicker ECM. Herrera et al. showed how PDGF-stimulated fibroblasts generated a thicker matrix as well as higher fiber orientation[7]. BJ-hTERT fibroblasts were incubated with or without PDGF and representative areas observed showed a more aligned cell distribution in matrices produced by PDGF-stimulated fibroblast (**Figure 1a**). Collagen I and fibronectin protein expression was increased in matrices derived from PDGF-stimulated fibroblasts (**Figure 1b**) and, consequently, matrix thickness was increased (**Figure 1c**). Moreover, collagen I and fibronectin show parallel patterns, as shown in directionality histograms (**Figure 1d**).

3D matrices derived from PDGF-BB-stimulated fibroblasts induce tubulogenesis in endothelial cells. HUVEC cells were seeded onto decellularized matrices derived from PDGF-stimulated or non-stimulated BJ-hTERT fibroblasts. Endothelial cells seeded on matrices generated by PDGF-stimulated fibroblasts showed more capillary-like structures than under non-stimulated conditions (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: PDGF-stimulated fibroblasts enhance thicker and anisotropic ECM. (**A**) Binary images representative of cellular orientation of the BJ-hTERT fibroblasts treated or not with PDGF-BB (above) and directionality histograms (below), which represent the frequency of distribution of cell angles (centering on the 0° angle). (**B**) Increase in protein expression of the extracellular proteins fibronectin and collagen I in PDGF-stimulated fibroblasts derived from ECM. (**C**) Increase in ECM thickness in those ECMs derived from PDGF-stimulated fibroblasts. (**D**) Increase in protein and organization of extracellular proteins such as fibronectin and collagen I in PDGF-stimulated fibroblasts derived from ECM. Below, directionality histograms. *$p < 0.05$; ***$p < 0.001$. Adapted from Herrera et al.[7]

Figure 2: PDGF-stimulated fibroblasts promote endothelial cell activation, which was observed by the formation of capillary-like structures on matrices derived from PDGF-stimulated fibroblasts. The “Particle analysis” tool from ImageJ showed an increase rate of 1.81 in HUVECs seeded on matrices from PDGF-stimulated fibroblasts regarding those seeded on matrices from non-stimulated fibroblasts. Adapted from Herrera et al.[7]

DISCUSSION:

Matrices can be generated with immortalized fibroblasts or primary fibroblast cultures. Fibroblasts are easy to maintain in in vitro culture with a high growth rate and stress resistance. They can even be isolated from post-mortem tissue[3], although contamination could be a restriction, depending on the tissue origin.

The 3D-matrix model here offers a novel and effective system to successfully study ECM composition and fiber orientation. It is also suitable for the analyses of fibroblast activation status, gene/protein expression, interactions with other cell types, etc. It is important to highlight that matrices generated with fibroblasts from patients make personalized study of

stroma-related mechanisms in tumors possible. For instance, this approach can be applied to studies of chemo-resistance, in which the ECM has an important role. The translation of this type of study could aid in decision making on the treatments of patients in clinical practice, implementing personalized medicine.

Even though the 3D-matrix protocol can be tedious, interesting results can be achieved by following instructions. It must be noted that multiple washing steps are needed, so avoiding matrix damage is essential. In addition, we recommend that when different cell culture conditions are tested with this protocol, matrices should be generated at the same time due to minimal differences that may occur during experiments (e.g., using the same fibroblast suspension when seeding pre-treated plates).

Other tissue-engineering methods are being developed, but additional steps like sterilization are needed to create compatible ECM and tissues for human use. Although new technologies are arising, they can be costly and may be difficult to adapt to standard laboratory facilities. The use of primary CAFs obtained from patients makes it possible to generate “personalized matrices” to test different treatments. Along this line, our group has been using this methodology to demonstrate that matrices generated by CAFs are different to the ones generated by NFs, showing thicker and more organized matrices[7]. We have reported that capillary-like structures derived from endothelial cells are dependent on fibroblast Snail1 expression, and there are future studies to observe that effect on CAFs-derived matrices.

ECM requires further study before we can understand the underlying mechanisms responsible for chemotherapy resistance or tumor relapse. Therefore, we suggest the use of CAF-derived matrices from cancer patient tissues as a procedure to investigate microenvironmental behavior in cancer and ECM communication with cancer cells or other stromal cells.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

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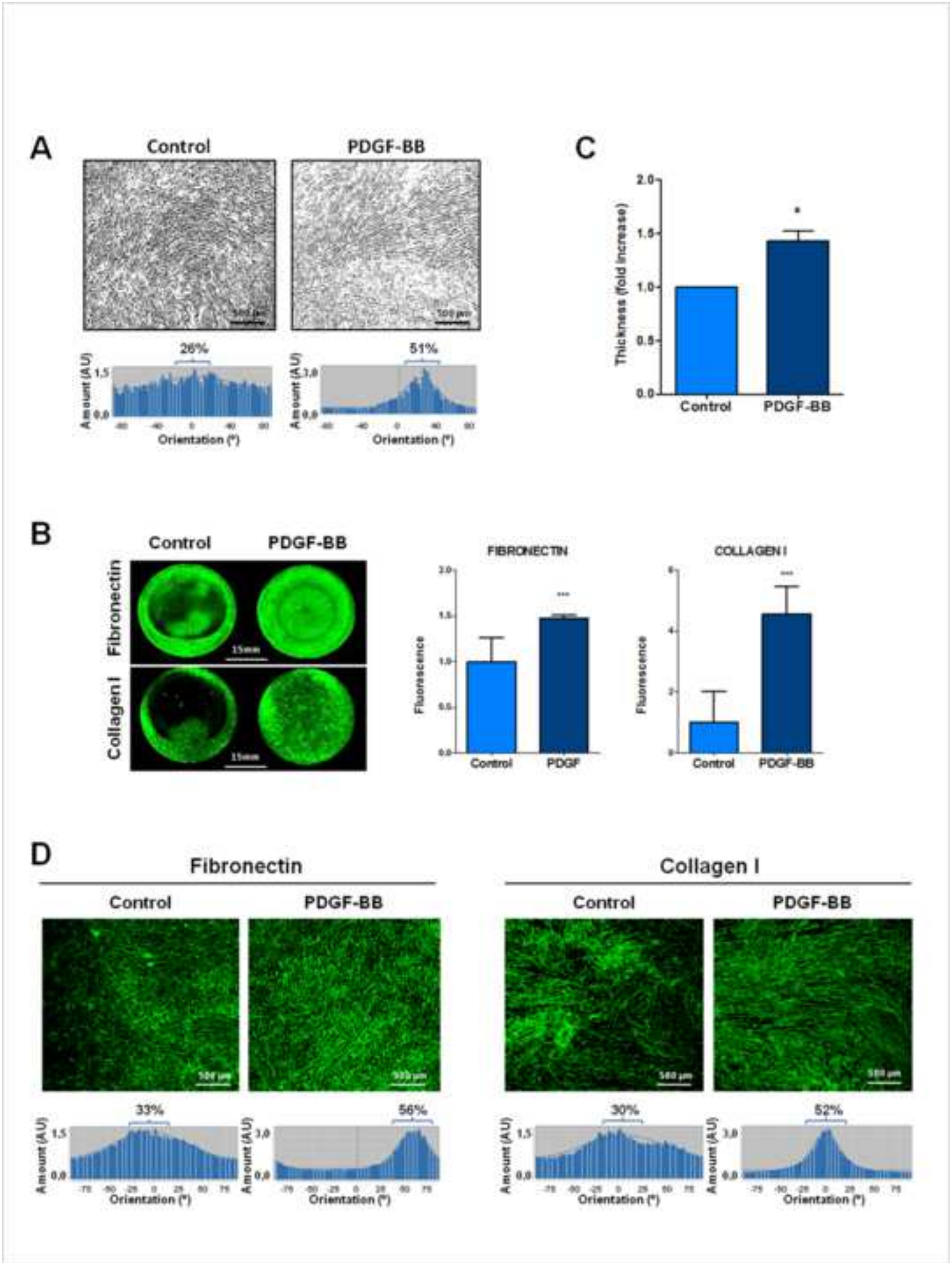
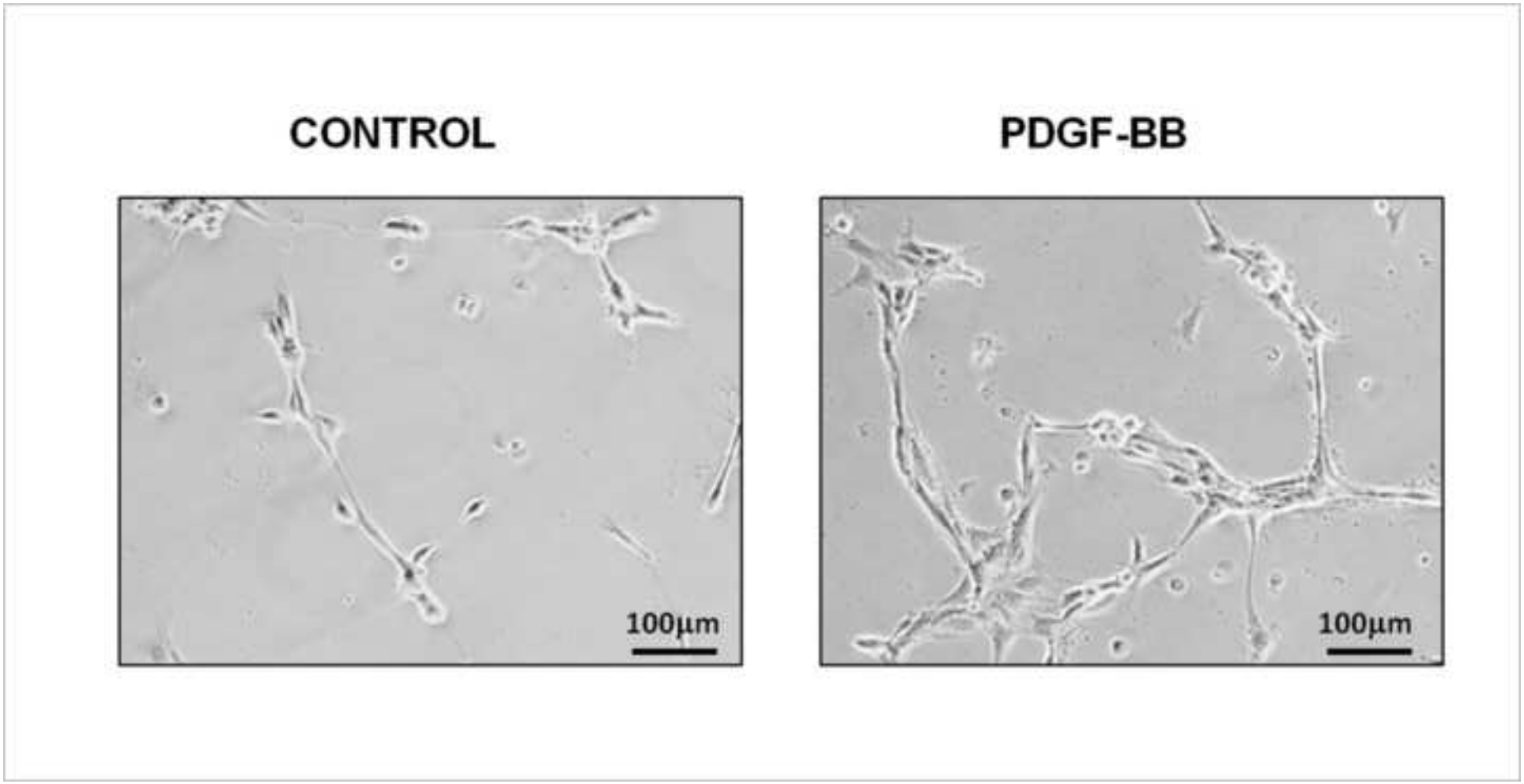


Figure 2



Name of Material/ Equipment	Company	Catalog Number
Ammonium hydroxide (NH4OH)	Roth	A990.1
Amphotericin-B	Corning	30-003-CF
Bovine Serum Albumin (BSA)	Sigma	A7906-50G
Dulbecco´s Modified Eagle Medium (DMEM)	Corning	10-014-CVR
Endothelial Basal Medium (EBM)	Lonza	CC-3121
Endothelial cell Basal Medium Supplements (EGM-2)	Lonza	CC-4176
Ethanolamine	Sigma	411000-100ML
Fetal Bovine Serum (FBS)	Biowest	S181B-500
Fibroblast Growth Basal Medium (FBM)	Lonza	CC-3131
Fibroblast Growth Medium Supplements and Growth Factors (FGM-2)	Lonza	CC-4126
Gelatin from bovine skin	Sigma	G9391-100G
Glutaraldehyde	Sigma	g5882
L-Ascorbic Acid	Sigma	A92902-100G
L-Glutamine	Lonza	BE17-605E
Normocin	Invivogen	3ANT-NR-2
Pencillin/Streptomycin (Pen/Strep)	Gibco	15140122
Phosphate Buffered Saline (PBS)	Corning	21-040-CVR
Triton X100	Roth	3051
Recombinant telomerase transfected immortalized human foreskin fibroblasts (BJ-hTERT)	ATCC	ATCC CRL-4001
Human umbilical vein endothelial cells (HUVECs)	ATCC	PCS-100-013

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3rd September 2018

To:

Dr. Phillip Steindel
Review Editor
JoVE

We are grateful for the constructive criticism of our manuscript, JoVE60304 "Fibroblast Derived 3d Matrices System Applicable to Endothelial Tube Formation Assay," by Galindo-Pumariño et al. We have attach a revised version addressing the issues raised by the reviewers' comments.

This new version has been edited and revised by a native English speaker to improve readability and understanding. We have add new quantification data, references and explanations to clarify some issues and to improve data presentation.

A detailed point-by-point summary of the revision is provided in the following pages.

We hope that you will find this revised version of the manuscript suitable for publication and look forward to hearing from you,

yours sincerely,

Cristina Peña

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Editor´s comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been revised by an English native speaker.

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

Format has been revised.

3. Please use periods for decimals (e.g., 0.05 instead of 0,05).

We replaced "." Instead "," in all decimals.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Matrigel, Normocin.

Matrigel TM symbol has been removed, although the word "Matrigel" is needed in the text.

Protocol:

1. If necessary, please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

A clarifying sentence has been added in protocol section 2.3

2. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Specific Protocol steps:
1. 2: Where do the cells in 2.1 and 2.2 come from? They are not in the Table of Materials.

ATCC references has been included in protocol sections 2.1 and 2.2 and in the Table of Materials.

2. 3.13: How many times total is this done? It looks like it can only be done twice, but the instructions seem to indicate 3 times (done once, then repeated twice). Please clarify.

The washing is done 3 times. A clarifying sentence has been added in protocol section 3.13

3. 4.1: Around how long will this take?

It will depend on culture conditions. HUVECs ATCC reference has been included in the text if maintenance guidance is needed.

Figures:

1. Please remove ‘Fig. 1’ etc. from the Figures themselves.

Labels Fig.1 and Fig 2 were removed from the corresponding figures.

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References has been revised and author list edited as indicated.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We have revised and filled with all information.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The manuscript deals with developing a natural extracellular matrix (ECM) developed from fibroblast cell line as can be useful for obtaining 3D cultures. Also, the authors describe the protocols to render the ECM almost cell-free and proved its usefulness for in endothelial cell tube-formation. The protocols detailed/described will be useful for the development of novel natural ECM matrices as can be used for a variety of applications in the areas of 3D cell cultures and tissue engineering. Surely, an useful contribution to the field.

We appreciate the generous comments from this reviewer.

Major Concerns:
Nil

Minor Concerns:
Nil

Reviewer #2:

Manuscript Summary:
Pena et al. propose fibroblast-derived 3D matrix that can be utilized for endothelial tube formation assay. In their protocol, they used immortalized BJ-hTERT fibroblasts to create 3D matrix and examined 3D matrix for capillary-like structure formation using HUVECs.

Major Concerns:

1. First of all, the data (Fig. 1) is exactly the same as published in Oncogenesis, 2018. I understand the publication policy of JoVE that is quite different from the other journals. Nonetheless, I feel this is an ethical issue. I think no article can carry the same results that was already published, even in the same research group.

Actually the scope of the JoVE is to deeply describe the methodology developed in previous research. Thus, the manuscript is formatted as a methodology paper as PubMed-indexed video article. In this way we may use previous results. However, based on the comments from reviewer three minor changes have been added to the figure 1.

2. In cell culture preparation, the authors noted three different cell types: BJ-hTERT, endothelial cells, and primary fibroblasts (CAF). However, the result (Fig. 2) just showed HUVECs on BI-hTERT derived matrix.

In the “Cell culture preparation” section, we just described the normal and standard procedure to describe the cells which will be used to develop the protocol. As they are ordinary procedures we did not show “Representative results”.

To generate the tube formation assay on fibroblasts-derived 3D matrix a fibroblasts cell line has to be cultured followed the culture of the endothelial cell line. In this way we described the conditions to culture the different cell lines but in the figure 2 we just represent, as representative results, the end point of the experiment in which the endothelial tube formation is observed.

3. The authors seem to be interested in developing a cancer-mimetic model in vitro. If so, what kind of implications the present data represent? Actually no data are provided using CAF.

In our previous studies, we described the validation of changes in ECM properties depending on SNAI1 expression and PDGF treatment using NFs and CAFs. In parallel as we commented in the current manuscript, we describe the reproduction of the most analogous in-vivo matrix which could be a valuable tool to investigate ECM’s role in angiogenesis or micro-environmental cell interactions.

We did not provide CAF-derived matrix data because we just described the methodology, which is the aim of the manuscript. We also think that conclusive studies on this topic are beyond the scope of the present manuscript. Anyway, we have introduced a sentence on page 7, line 274 to comment our CAF data.

4. I do not understand why we need thicker matrix for endothelial tube formation assay? There is an early report that demonstrate the formation of capillary-like structure on fibroblast-derived matrix (Tissue Eng Part A 2014, 20(17-18):2365-77)

This comment appears to be based on a misunderstanding. The data presented in this manuscript as “Representative results” are those referred to Herrera et al, Oncogeneisis 2018. In that study, we described the orientation and the parallel alignment of the extracellular matrix fibers as the main cause of endothelial tube formation. Certainly, this anisotropic fibers are accompanied to the generation of a ticker ECM.

Moreover, in the cited manuscript (Tissue Eng Part A 2014, 20(17-18):2365-77) the authors observed a very active response of endothelial cell on matrix derived from fibroblasts or preosteoblast, but not on matrix from chondrocytes. Regarding the compositional and physics differences in those matrix which could be related with this different endothelial cell response the author argued the tissue origin of primary cells (vascular/avascular), production of macromolecules, residual growth factors, matrix elasticity, and, as we similarly described, matrix thickness. Thus, in line with our data, the authors observed that matrix derived from chondrocytes was much thinner than those derived from fibroblasts or preosteoblast. We have introduce a sentence on page 2, line 80, referring to this data/manuscript.

Minor Concerns:

1. There is no explanation about using 1% glutaraldehyde and 1M ethanolamine during fibroblast derived 3D matrix preparation.

We have added an explanation of 1% glutaraldehyde and 1M ethanolamine use in protocol sections 3.3 and 3.5, respectively.

Reviewer #3:

Manuscript Summary:
This manuscript describes a methodology for generating fibroblast derived 3D matrices to then examine how different matrix conditions affect endothelial tube formation. Overall, this methods manuscript is presented in a sequential manner that is easy to follow. The figures are well set out with appropriate font sizes and labels, however further statistical analysis could be performed in some cases to aid understanding. I am concerned that the methodology lacks novelty as most of the protocol is published elsewhere (Ref 15: Castelló-Cros, R. and E. Cukierman). Also, there are some minor language issues with this manuscript. This paper should be edited and revised by a native English speaker to improve readability and understanding. Overall, with minor revisions, this manuscript and subsequent video would be of great interest to the reader/viewership of JoVE.

Major Concerns:

1. No mention of PDGF-BB treatment in the protocol section yet all the figures relate to this. Please include a section in protocol where you would stimulate the fibroblasts with PDGF-BB or other growth factors/reagents.

A clarification sentence has been added in protocol section 3.10, as grow factors would be added at the same time as ascorbic acid treatment.

2. Please perform statistics (e.g. non-parametric Mann-Whitney U tests) on all orientation data supplied in Fig 1.

In statistics, the non-parametric test Mann–Whitney U test is a test of the null hypothesis that it is equally likely that a randomly selected value from one sample will be less than or greater than a randomly selected value from another independent second sample having the same distribution.

To study the anisotropy of our samples we could have images with high anisotropy but with a totally difference angles orientation between both samples. For instance, a picture with all fibers presenting and orientation at 0º, and another picture with all fiber showing an orientation of 90º. In this case the Mann–Whitney U test, would offer a very statistical significant difference with a p value really low. However, both samples would present a very similar anisotropy. In this way we cannot perform this kind of statistical test.

A quantification procedure to show differences in the anisotropy of the samples/pictures could be based in the quantification of the percentages of orientated fibers accumulated in a range of ±20º around the modal angle (Stanisavljevic et al, Cancer Res 2014). We have performed this calculation and this data are now presented in the figure 1A.

3. Please quantify superior endothelial tube formation in Fig 2 using Image analysis software. Could analyse total/avg vessel length, avg branching per vessel etc.

Due to the small size of the pictures it is difficult to calculate total/average vessel length or average branching per vessel. However, following the suggestion of the reviewer we have quantified the images by analyzing the particles (Particle Analysis tool from Image J software) to quantify vascular cells. This data are now added in the figure legend of figure 2.

Minor Concerns:

1. The resolution of the figures is poor, please improve for publication.

We have improved the resolution of the images as JoVE guidelines requires.

2. Please provide scale bar on Fig 1b images.

The scale bar has been added to the image.

3. Spelling mistake at Line 35: "pro-tumorogenic roles"; Line 66: "EMC"; Line 77-78: "Furthermore, CAFs and ECM are involved in the vascular system, both in vase generation and as part of the vase outer layer". Grammatical error at Line 53, 88. Please proofread the manuscript.

The manuscript has been edited and revised by a native English speaker to improve readability and understanding as the reviewer suggested.

4. Reference required at Line 50: "Fibronectin generated by fibroblasts is needed for cell adhesion".

The reference has been added.

5. Need to write acronyms in full first before using in both manuscript and protocol. Eg: Vascular endothelial growth factor (VEGF).

TGF-β full named in line 61

MMPs full named in line 78

VEGF full named in line 83

PDGF full named in line 84

TNF-α full named in line 92