공학석사학위논문

주변세포로 감싸진 체외혈관 제작 및 특성

Engineering and characterization of pericyte covered blood vessel in vitro

2014 년 8 월

서울대학교 대학원

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Abstract

Engineering and characterization of pericyte covered blood vessel in vitro

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Neovessel formation is composed of several steps, but most preliminary studies exploited only endothelial cells for generating *in vitro* microvasculature, which has not successfully reproduced the last stage, vessel stabilization. It is well known that vessel stabilization is conducted by pericytes which wrap around the vessel wall. Moreover, it is known that pericytes are involved in cancer metastasis and vascular diseases such as diabetic retinopathy, playing a critical role. Therefore, in this study, we generated vascular networks morphologically, functionally more similar with *in vivo* through co-culture of endothelial cells and pericytes in a microfluidic device.

Surprisingly, 3 days later of seeding mixed pericytes and endothelial cells suspension, pericytes lagging behind vascular sprouts were observed, which was seen *in vivo*. And after 6 days, we confirmed smaller vessel width and more complex features when co-culturing than when culturing only endothelial cells. Also, in order to compare the functional ability, 4 kDa and 70 kDa FITC-dextran were introduced into the vessel. There was no perceptible difference when introducing 70 kDa FITC-dextran, whereas vessels associated with pericytes showed markedly lower permeable tendency which was shown at normal condition of *in vivo* when introducing 4 kDa FITC-dextran. This system can be used for mimicking several diseases caused by dysfunctional pericyte or basis of blood brain barrier as well as pharmaceutical assay

Keywords : pericyte, microfluidics, coculture, characterization, perfusion

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Chapter 1. Introduction

Neovascularization is a multistep process [1]. In angiogenesis, formation of vascular sprouts from pre-existing vessels, mural cells such as smooth muscle cells or pericytes are associated with vessel wall in the end of the process, stabilizing the vessel [2]. Especially pericytes are deeply involved in some vascular diseases due to their role. Above all, diabetic retinopathy or cancer angiogenesis has been known to be closely related with aberration in endothelial cell (EC)-pericyte interactions. In the case of diabetic retinopathy, pericytes drop out from the vessel wall induced by hyperglycemia at the early stage of disease, and this morphological change leads to vessel leakage and eventually blindness [3-6]. Pericytes also wrap around tumor-associated vasculatures. Although it is generally sparser than normal vasculature, VEGF-A secretion from surrounding pericytes makes EC viability high, lowering the success rate of anti-VEGF therapy. In order to raise the effect of anti-cancer therapy, attempts to target both pericyte and vessel simultaneously have been made [7, 8]. Thus, as importance of the pericyte role has increasingly grown, several attempts to coculture ECs and pericytes have made for the purpose of engineering more physiologically, functionally similar vascular networks during in the past 10 years.

However, no method conducted previously has emulated *in vivo* vascular networks perfectly. Commonly used *in vitro* method such as transwell assay has advantages like experimental simplicity and high throughput. Nevertheless, it has limitation with mimicking the microenvironments where 3D cell-cell and cell-extracellular matrix (ECM) interactions exist because cells are cultured onto the 2D porous membrane [9]. In order to overcome the limitation of 2D, several approaches to construct 3D environments for cell culture using ECMs such as fibrin, collagen and matrigel has been made. In these approaches such as bead assay with ECs and pericytes attachment [10], embedding EC/pericyte cell suspensions [11-13] and mixed EC-pericyte spheroids inside hydrogel [14], vascular networks associated with pericytes were formed but they were not able to show vascular perfusability which is the most important function of vessel.

The aim of the present study is to engineer physiologically relevant with *in vivo* vascular networks as well as having physical function such as barrier role, substance transfer pathways by successful coculture of ECs and pericytes in a microfluidic device. Functional and morphological similarity of pericyte covered blood vessels with *in vivo* will be verified by comparing vasculatures with only ECs and ECs/pericytes through characterizing of several vascular properties.

Chapter 2. Methods and materials

2.1 Microfluidic system

Microfluidic devices were fabricated by photo lithography and soft lithography. Microfluidic channels were designed by Auto CAD depending on the experimental purpose. In this research, device design is same with former one which was previously designed by our group. Microfluidic devices were made with mixture of Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) elastomer and curing agent. PDMS and curing agent were mixed in the ratio of 10:1 (w/w) and this was poured onto the previously fabricated master mold and degassed under a vacuum chamber. Degassed PDMS was cured on the 85℃ hot plate for 30 min to 1 h. After hardening, it can be peeled off from the master mold and four inlet and outlet reservoirs were punched by a 6mm biopsy punch and four small holes for fibrin gel and fibroblast-gel mixture injection port were punctured with syringe needle. The PDMS devices and coverslips were cleaned with adhesive tape, and then were permanently bonded by oxygen plasma treatment.

2.2 Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs, Lonza) were cultured in endothelial growth medium (EGM-2, Lonza) with full supplements and were used at passage 4. Dermal fibroblasts (DF, CEFO) were grown in Fibroblast growth medium (FGM-2, Lonza). Human pericytes from placental (hPC-PL, Promocell) were cultured in pericyte growth medium (PGM, Promocell) and all were used at passage 6. All cells were kept in a humidified incubator at 37℃ and 5% CO_2.

2.3 Angiogenesis assay

The angiogenesis assay protocol is almost identical to the method shown in previously published paper in our group [15]. Fibroblasts were detached from the cell culture dish and they were mixed with 2.5mg/ml fibrinogen solution (2.5mg/ml fibrinogen, 0.15U/ml aprotinin) at a cell concentration of 10^7cells ml^{-1}. Fibroblast suspension was injected into the right side fibroblast channel as soon as this gel suspension was mixed with thrombin (0.5U/ml). After 30 minutes, 2.5mg/ml fibrinogen (2.5mg/ml fibrinogen, 0.15U/ml aprotinin) mixed with thrombin (0.5U/ml)

was put into the central channel. After polymerization, coculture media (EGM-2) filled upper reservoirs in each device and in order to make media channels hydrophilic, that media was sucked slightly on the lower reservoirs. The devices were incubated at 37℃ over a day to dissipate the air bubbles formed between gels and media interface. HUVECs were suspended at a cell concentration of $6x10^6$cells ml^{-1} and in case of coculture with pericyte, HUVECs ($6x10^6$cells ml^{-1}) and pericytes ($1.2x10^6$cells ml^{-1}) were prepared in the ratio of 5:1. Media in each reservoir were all removed and 5 μl cell suspension was put into the media channel contralateral to the fibroblast channel. And then device was tilted to 90 degree for about 30 minutes in an incubator to attach cell mixtures to the gel-media interface. 30 minutes later, all reservoirs were filled with media.

2.4 Immunostaining

Samples were fixed with 4% paraformaldehyde (Thermo) for 15 minutes and then permeabilized by 0.15% triton-X 100 (Sigma-Aldrich) for 20 minutes at room temperature. After permeabilization, cells were blocked by 3% bovine serum albumin (BSA, Sigma-Aldrich) for an hour at room temperature. After washing three times

with phosphate-buffered saline (PBS, Hyclone), samples were incubated overnight with Alexa Fluor 647 conjugated mouse monoclonal CD 31 antibody (Biolegend) as a vessel marker and Alexa Fluor 488 conjugated mouse monoclonal α-SMA antibody (R&D systems) as a pericyte marker. In case of nucleus staining, samples were incubated for 2 hours with hoecst 33342 (invitrogen) at 1: 1000 dilution. After all, Samples were washed three times with PBS and all reservoirs were filled with it, and then preserved in 4℃ refrigerator before imaging.

2.5 Microscopy and imaging

For obtaining 3D construction of vessels and cross sections, images were taken by confocal microscope (Olympus FV1000). All images were captured with a 20x lens. An instance in measuring permeability, IX81 inverted microscope (Olympus) was employed to take images.

2.6 Data analysis

All data was analyzed by Fiji (http://fiji.sc/Fiji). All sample images were taken

by confocal microscope and composed of about 55 stacks. Before analyzing the vessel width, area and the number of branch points, confocal data should be converted to 2D binary image. Confocal images of a sample composed of about 55 stacks largely comprise a lower and an upper vessel networks, so instead of z-projecting all the stacks of data, sample was z projected in twice to minimize the counting error. Counting error means that potential occurrence of error especially in counting the number of branch points in a case like that two vessels which are existed in skew position might make a branch node when they were z projected once. In the process of converting projection images into binary images for data analysis, Gaussian blur and despeckle were preceded to make an image smoothing and reduce the background noise. After image pre-processing, threshold was applied to each projection image in accordance with their original 3D images. ROI (580.50x1769.32 μm^2) was set excluding the starting and ending part of the image where so many variables exist. Skeletonize plugin in Fiji was used to calculate the number of branch nodes and branches. In case of vessel width, designated width of ROI was divided into three equal parts, and among all y values of every one-third, two-thirds portion from origin where the following point value changes such as 0 to 255 or 255 to 0 could be found through processing 2 program (http://processing.org/).

So, vessel width could be calculated by subtracting the sequential y values of detecting points. Some calculated values not included in definition of vessel width were excluded. Vessel width is defined as the case which line connecting the sequential y values representing the length of white parts pass the line of skeleton only once. Vessel area could be computed the extents where white parts occupy.

2.7 Permeability measurement

Permeability could be observed by introducing 4 kDa, 70 kDa FITC-dextran (Sigma-Aldrich) into the entrance of microvasculature. Media was aspirated from the reservoirs and then FITC-dextran solution was put in the one of four reservoirs. When vessels were perfused, small molecules went into escaping from intravascular to extravascular region as time went by. Sequential images were taken by IX 80 inverted microscopy in 20 second intervals over a period of 5 minutes and permeability could be compared through extravascular regional intensity change.

2.8 Statistical analysis

Error bars mean standard error. Statistical significance was defined as $p<0.05$ and evaluated by Student's T-tests. All data were analyzed by Sigma plot 10.0.

Chapter 3. Results

3.1 Experimental procedure

Same design of the microfluidic chip from the previous study our group exploited was used [15]. (Figure 3.1.) In this research, we tested only angiogenesis procedure, and fibroblasts were injected only into the right side fibroblast channel. Although most previous *in vitro* microvascular systems utilized lung fibroblast (LF) in order to induce neovessel formation from ECs [16], LFs failed to make physical association between ECs and pericytes in case of coculture. (Figure 3.2) It is inferred that LFs are widely known as a huge VEGF-A secretor, but in vessel maturation process, VEGF-A is not much necessary unlike in angiogenesis initiation process [17]. Thus, dermal fibroblasts (DFs) were used in this system instead of LFs. EC and Pericyte mixture at the ratio of five to one was attached at the left side of the central acellular matrix. Sprouts of ECs extended toward fibroblasts, followed by pericytes. 3days after seeding cells, ECs grew up to the opposite end of the central channel but lumens were not formed properly and vessel width was quite thin. When DFs were used, formation of perfusable vessel networks took one more day compared to the

experiments with using LFs. This means after 6days, perfusable vessel networks which can be a path between two media channels were made and pericytes were normally adhered to over the half of the vessel networks. (Figure 3.3)

3.2 Morphological difference between EC monoculture and EC/pericyte cocultures

In order to confirm the morphological difference between EC monoculture and EC/pericyte cocultures, 3D images were taken by confocal microscopy. Also, pericyte coverage of the abluminal endothelial surface could also be confirmed. (Figure 3.4) Figure 3.4A shows vascular networks made by ECs, while Figure 3.4C by ECs with pericytes. Figure 3.4B and Figure 3.4D display the magnified image of white dotted box in Figure 3.4A and 3.4C, respectively. As shown by Figure 3.4D, pericytes were associated with vessel walls which can be identified by cross sectional views (white arrows). Through vasculature binary mask images converted by 3D stack images, we can get the quantitative data for vascular network properties as well as distinguish between differences from qualitative data. First, region of interest (ROI) was divided into three even parts and vessel widths were measured and compared at each section.

Vessel networks comprised of ECs and pericytes had 33.0±0.86 ㎛ diameter at a third of length of ROI width apart from origin and 40.6±1.84 ㎛ at two thirds of ROI, while networks comprised of only EC had significantly higher width at both two positions: 46.3±1.15 ㎛ and 47.5±2.15 ㎛ respectively. (Figure 3.5) This demonstrates the fact that pericytes block the vessel enlargement. Furthermore, samples cultured during 6 days were used for analysis but protective effect of vessel dilation in coculture was more specific when keeping cells over two weeks. Also, EC/pericyte cocultures showed more intricate features in terms of number of branches and junctions. (Figure 3.6) EC monoculture condition had 50.1±1.14 junctions and 79.6±2.15 branches, while EC/pericyte coculture condition showed nearly 50% increase in number of junctions (83.54±0.86) and branches (131.9±1.84). Also, using these features, vessel density was measured by dividing the number of junctions by vessel area. (Figure 3.7) Coculture condition showed denser vascular network features with more branching, resembling more of vascular developmental procedure in nomal conditions.

3.3 Functional difference between EC monoculture and EC/pericyte cocultures

For validating the functional ability of engineered vessels, FITC-dextran with molecular weights 70 kDa and 4 kDa, were introduced into the entrance of vascular networks. 70 kDa FITC-dextran is widely used for vascular permeability test because it has a similar molecular weight with albumin which is one of the most abundant proteins in plasma. With 4 kDa FITC-dextran, permeability change can be quickly and easily observable due to its lighter molecular weight. After introducing FITC-dextran, gradual escape from vascular compartments to extra-vascular compartments was observed. There was no remarkable leakage of 70 kDa FITC-dextran for 320s in both conditions, whereas clear leakage of 4 kDa FITC-dextran was observed. Moreover, vascular networks formed only with ECs showed higher permeable tendency than that of ECs with pericytes after equal time has passed. (Figure 3.8)

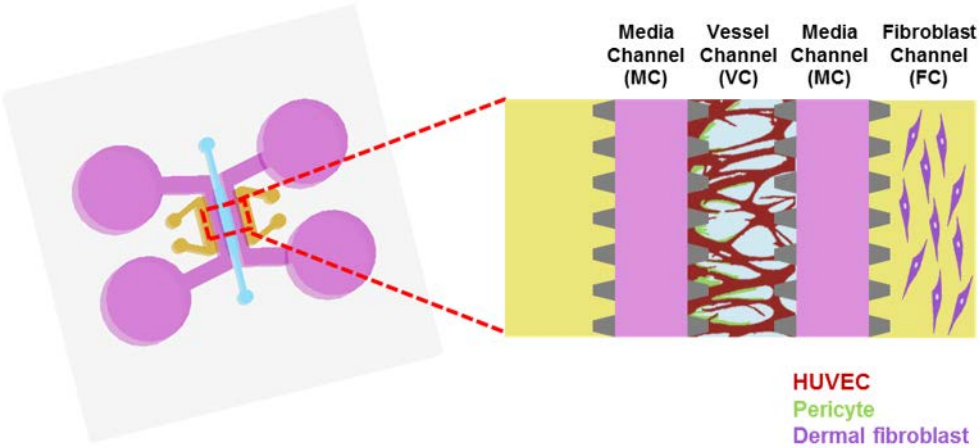

Figure 3.1 Schematic of the microfluidic system. Microfluidic device is composed of central vessel channel, two adjoining media channels and the outermost fibroblast channel. Vascular networks (red) covered by pericytes (green) was formed in the vessel channel and this was induced by fibroblasts (violet) which secrete angiogenic factors and communicate with other cell types.

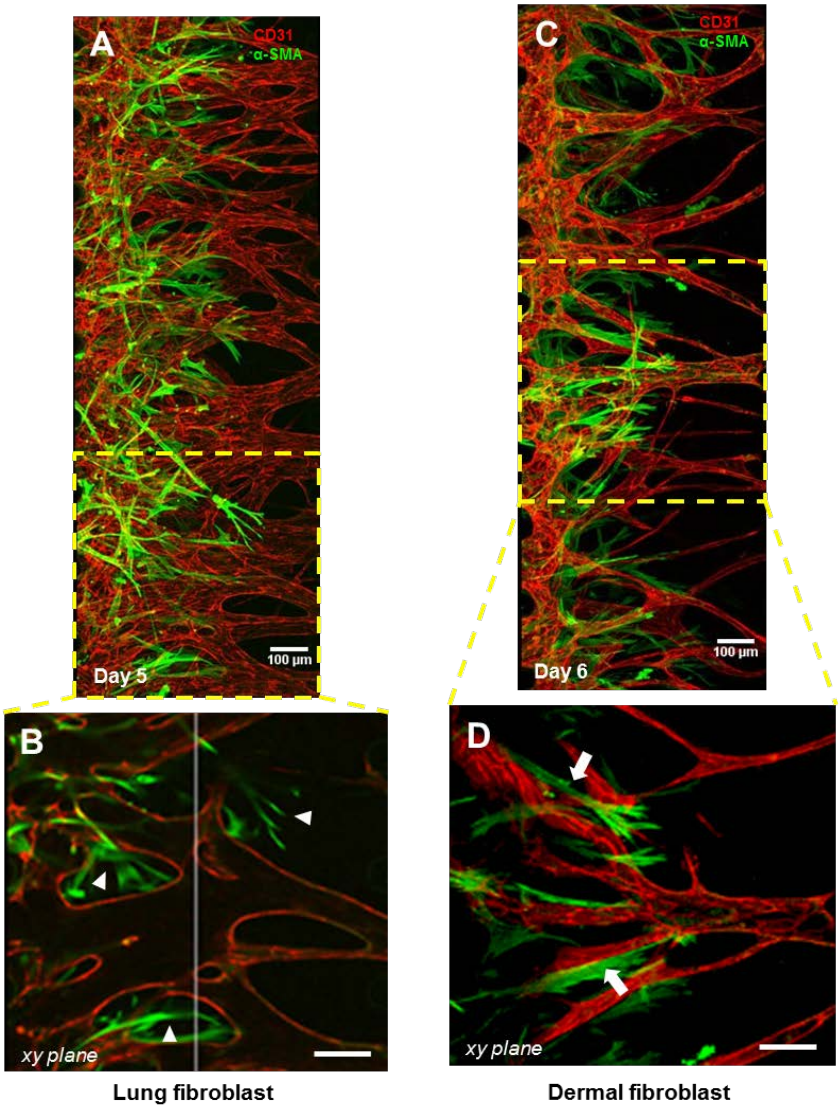

Figure3.2 Different appearance of interactions between ECs and pericytes depending on fibroblast type. Microvascular networks induced by (A) lung fibroblast and (C) dermal fibroblast. (B) Enlarged photograph of xy cross sectional plane of yellow dotted box in (A). Some pericytes did not adjoin the vessel networks. (White arrowheads) (D) Enlarged photograph of xy cross sectional plane of yellow dotted box in (C). Most of pericytes adjoined the vessel networks well. (White arrows) CD 31 (red), pericytes with α-SMA (green). Scale bar, 100㎛

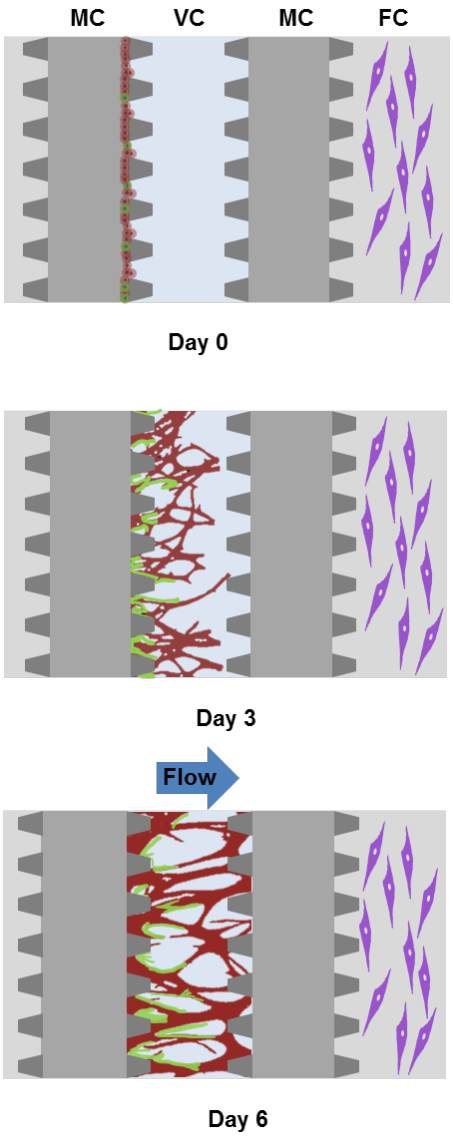

Figure 3.3 Schematic diagram of sequential procedure of vascular networks formation associated with pericytes. ECs and pericytes mixture with 5:1 ratio is attached on the left side wall of the vessel channel which was already filled with fibrin gel on day 1 early. Fibroblasts and fibrin gel mixture was injected into right side FC channel also on day 1 early. EC sprouts and pericytes followed behind are growing toward fibroblasts and after 6 days, perfusable vessel networks surrounded by pericytes are formed.

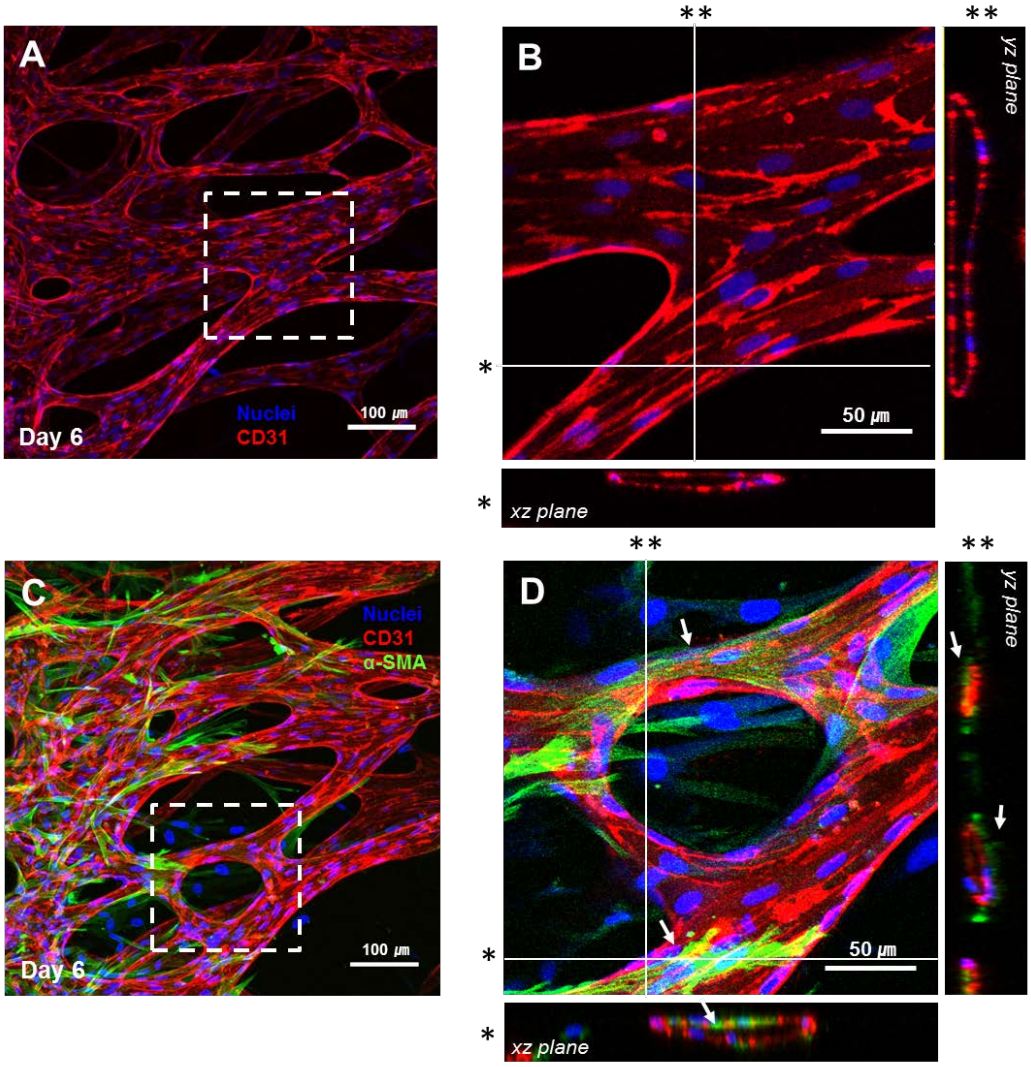

Figure 3.4 Two types of engineered vacular networks typified by immunofluorescence. Microvascular networks composed of (A) only ECs and (C) ECs with pericytes. (B) Enlarged photograph of a white dotted box in figure (A). (D) Enlarged photograph of a white dotted box in figure (C). After 6days of seeding cells, pericytes cover the blood vessels well (white arrows in D). Microvascular networks were stained with CD 31 (red), nucleus with hoecst (blue), pericytes with α-SMA (green). Scale bar, 100㎛

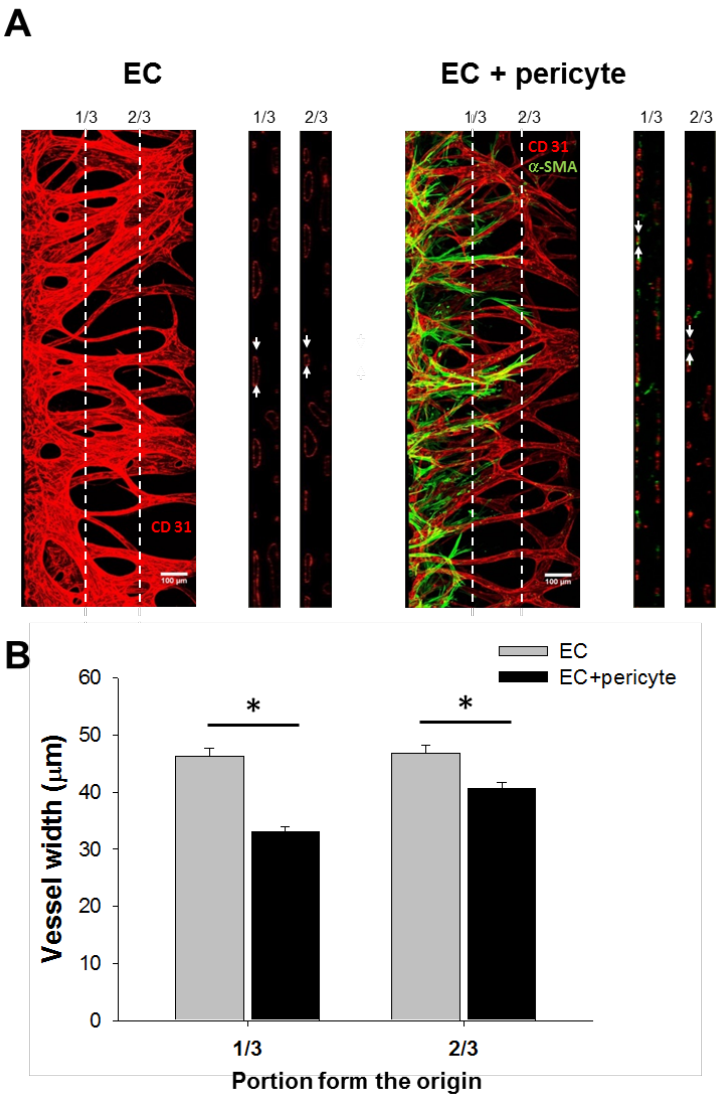

Figure 3.5 Coculture of ECs with pericytes changes the morphological characteristics of vessel width. (A) Width of ROI is divided into three equal parts. Cross sectional view of each portion shows vessel lumen. Arrows show different vessel width between EC and EC/pericyte cocultures. (B) Vessel widths were measured from z-stack binary images taken by confocal micrographs and they were quantified at one-third and two-thirds portion. When two cells were cultured together, significant decrease of vessel width was verified. Statistical significance, $P<0.05$.

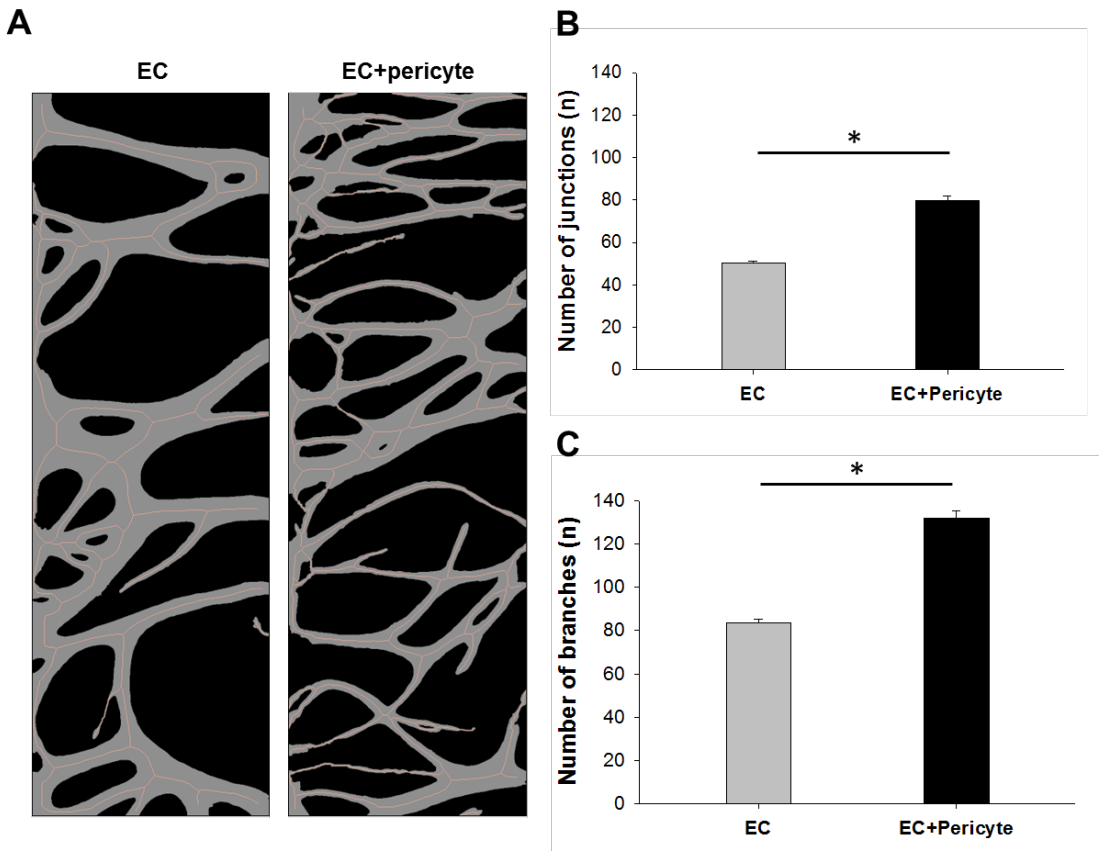

Figure 3.6 Characterization of the number of junctions and branches and comparison between EC monoculture and EC/pericyte cocultures. (A) Skeletonized images of each condition. (B) Number of branches and (C) number of junctions were measured from z-stack binary images. Number of junctions and branches are dramatically increase in coculture condition. Statistical significance, $P<0.05$.

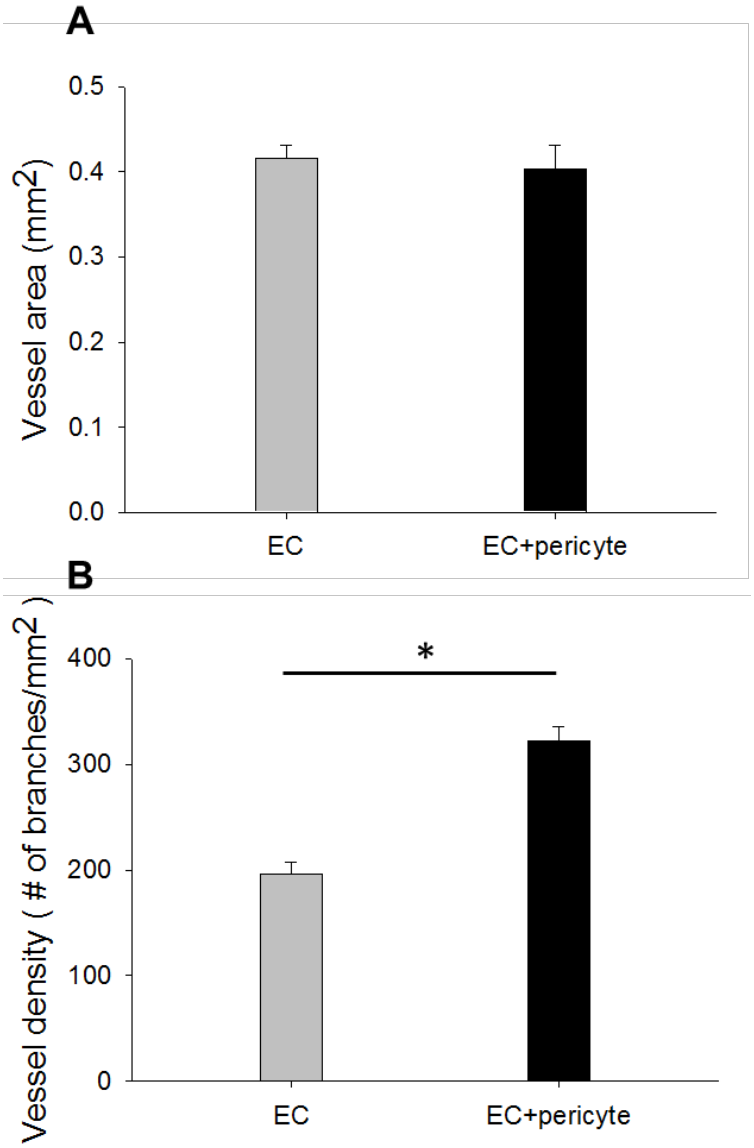

Figure 3.7 Characterization of vessel area and density and comparison between EC monoculture and EC/pericyte cocultures. (A) Vessel area and (B) vessel density were estimated from z-stack binary images. Vessel density dramatically increases in coculture condition but area occupied by vessel networks makes no difference. Statistical significance, $P<0.05$.

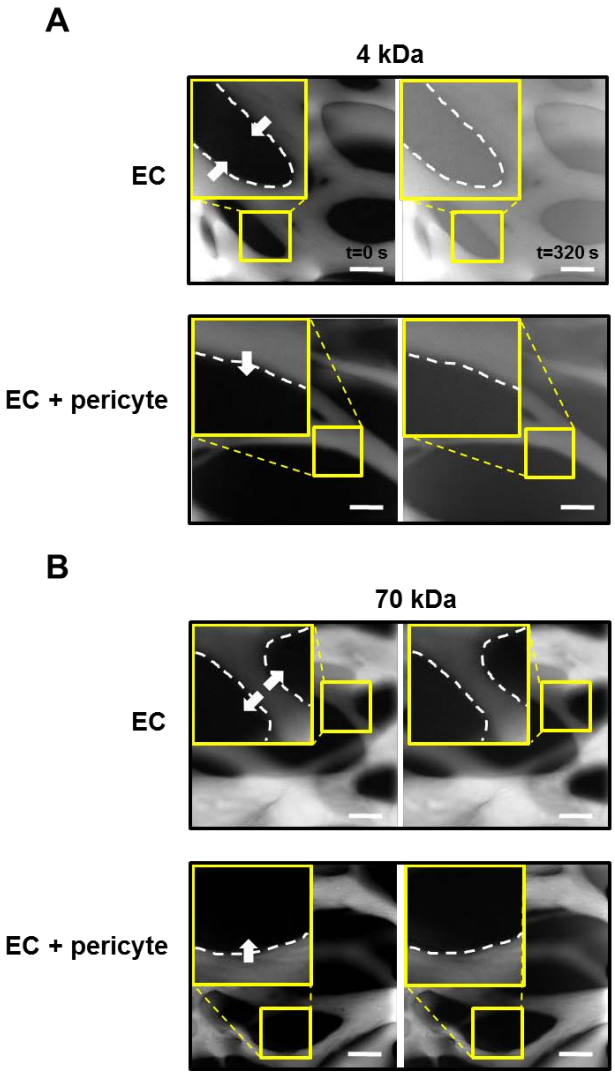

Figure 3.8 Permeability difference between EC monoculture and EC/pericyte coculture conditions. (A) With 4 kDa FITC-dextran introducing, vasculatures comprised of only ECs show significantly more permeable tendency than that of ECs with pericytes. (B) With introducing 70 kDa FITC-dextran, vasculatures comprised of ECs as well as ECs with pericytes demonstrate low permeability. White dotted line reveals boundary between vascular and extravascular compartments. White arrows indicate the direction for FITC-dextran escape. Scale bar, 100㎛

Chapter 4. Discussion

The objective of this study was engineering pericyte covered blood vessels by coculture of ECs with pericytes in a microfluidic chip. In most previous attempts to make a pericyte covered vasculatures in 3D, mixture of two kinds of cells embedded in hydrogel could neither mimic the sequential angiogenic process, neovessel formation followed by pericytes, nor make perfusable vessels which is one of the pivotal roles of vascular networks [10-14]. However, surprisingly, vasculatures formed by culturing the mixture of two types of cells within a microfluidic chip could embody both naturally mimicking the sequential steps of pericyte association with vessels and making perfusable vessels. After 3days of seeding cell suspensions composed of ECs and pericytes mixture, EC sprouts that had incomplete lumen grew up to the end of the vessel channel and pericytes lagged behind them, wrapping around EC sprouts. Finally, 6 days later, perfusable vascular networks covered with pericytes were made. This was mimicry of real phenomena shown on angiogenesis procedure [18, 19]. Although another research using microfluidic chip with different channel design made perfusable vessels, these were made artificially by following the

channel structures and also displayed a different feature shown *in vivo*, pericytes existing in the inner vasculature [20]. So, our method has great significance in terms of mimicking actual process which was not reproduced in previous researches [9-14, 20].

Although most studies still have concentrated on making vascular networks by culture of ECs alone [15, 21-23], pericytes are required in order to mimic the actual vessels *in vivo*. They control the vascular permeability and are also involved in blood flow regulation as well as vascular stability through physical and chemical interactions with endothelium [19, 24]. According to their fundamental roles, aberration in EC–pericyte interactions occurs in the early stage of various diseases and this gives rise to a functional disorder and abnormal vascular morphology [6, 25]. Normal vasculatures have smaller vessel widths by pericytes envelopment and more branches and junctions through branching than abnormal vasculatures as goes from proximal to distal section in terms of morphology [17, 25]. Permeability is also relatively lower than abnormal condition in accordance with tight and adherens junctions between ECs and pericytes. In this study, vascular characteristics such as vessel width, area, density and the number of branches and junctions were quantified from morphological analysis and compared between EC monoculture and EC and

pericyte cocultures in a microfluidic chip. As a result, characteristics shown in blood vessels *in vivo* such as smaller vessel width, more branch and junction number and higher density were verified in the coculture condition. These results were in close agreement with those of previous researches [12, 26], supporting the fact that pericytes participate in the regulation of vessel width and branching. [17, 25] Although most previous studies have succeeded in making *in vitro* microvascular networks which had similar morphological characteristics with *in vivo*, they failed to display functions such as perfusion, barrier function [10-14]. We identified the barrier functions as well as substances passing through the lumen by permeability measurements. When 70 kDa FITC-dextran infused into the vessels, diffusion of them to the extravascular region was insignificant in EC monoculture as well as EC-pericyte cocultures. Difference between two conditions was hard to identify with live images. However, when 4 kDa FITC-dextran which is much lighter than 70 kDa infused, vasculature with ECs showed higher permeable tendency.

So, we successfully generated vascular networks that are physiologically and functionally more alike with *in vivo* in a microfluidic chip. However, although vascular networks generated within this system are perfusable, it is impossible to make flow consistently. This can be solved by applying pump systems to microfluidic

device. In the future, it can be applied to study on the effect of flow on vessels and pericyte using advanced microfluidic systems.

Chapter 5. Conclusion

In the present study, we made *in vitro* microvascular networks more similar to *in vivo* through coculture of ECs with pericytes in a microfluidic device. We showed that microvascular networks associated with pericytes had smaller vessel width, complex branching features and lower permeability as compared with vasculature with no pericytes. This system, also, will sheds light on not only mimicking pericyte relevant disease model by detaching pericytes from blood vessel wall using certain growth factors or drugs but also simulating the blood brain barrier(BBB) microenvironment where various cells such as pericytes, EC, astrocytes interact with each other.

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초 록

신생혈관형성은 여러 단계를 거쳐 진행되지만, 내피세포만을 이용한 대다수의 기존 연구들은 마지막 단계인 혈관 안정화를 성공적으로 재현해내지 못했다. 혈관 안정화는 혈관벽을 감싸는 주변세포에 의해 이루어진다. 게다가 주변세포는 암전이와 당뇨망막병증과 같은 혈관 질병에 관여한다고 알려져 있기에, 그들의 중요성은 점차 증가되고 있다. 본 연구에서는 미세유체 소자 내에 내피세포와 혈관세포를 공배양하여 형태학적, 기능적으로 실제와 유사한 혈관망을 만들었다. 놀랍게도 내피세포와 주변세포의 혼합 현탁액을 미세유체 소자 내에 배양한 지 3 일이 지난 후, 실제 혈관에서 보여지는 현상과 유사하게 혈관망을 뒤따라 주변세포가 자라나오는 것을 관찰할 수 있었다. 또한 관류 가능한 혈관이 생성되는 6 일차에, 두 가지 세포를 공배양한 경우 혈관 내피세포만 배양했을 때보다 작은 혈관너비와 복잡한 혈관형상을 갖는 것을 이미지 분석을 통해 확인했다. 또한 두 조건 사이의 혈관의 기능적 차이를 비교하기 위하여 4 kDa 과 70 kDa FITC-dextran 을 혈관에 관류시켰다. 70 kDa FITC-dextran 의 경우 뚜렷한 차이가 발견되지 않았지만, 4 kDa FITC-dextran 의 경우 주변세포에 의해 감싸진 혈관은 실제 정상

혈관에서처럼 상대적으로 낮은 투과성을 보였다. 간단하면서도 실제와 높은 유사성을 갖는 혈관을 구현한 이 시스템은 주변세포 이상에 의한 질병 모델, 혈관 뇌 관문의 모사 및 약물 에세이로 사용될 수 있을 것이다.

주요어 : 주변세포, 미세유체소자, 공배양, 특성화, 관류

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