의학박사 학위논문

Comparison of calcification and transcriptome represented between peripheral vascular smooth muscle cells of end-stage renal disease patients with peripheral artery disease and normal aortic vascular smooth muscle cells under high phosphate condition

신장 질환으로 인한 말초혈관 질환 환자의 말초 혈관 평활근 세포와 정상 대동맥 평활근 세포의 고인산 환경에서의 석회화 비교 및 RNA sequencing을 통한 발현 전사체 차이 분석

2021년 8월

서울대학교 대학원
의학과 정형외과학 전공

이 동 오

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이동오의 박사 학위논문을 인준함

2021년 7월

위 원 장 이 재 협

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Abstract

Comparison of calcification and transcriptome represented between peripheral vascular smooth muscle cells of end-stage renal disease patients with peripheral artery disease and normal aortic vascular smooth muscle cells under high phosphate concentration

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Purpose: The role of peripheral vascular smooth muscle cells (VSMCs) in vascular calcification, which is related to chronic kidney disease (CKD) and severe peripheral arterial disease, has been overlooked compared to that of major VSMCs. The purpose of this study is to identify the calcifying characteristics, and to investigate differential expression genes (DEGs) of peripheral VSMCs in patients with critical limb ischemia (CLI).

Material and Methods: We isolated peripheral VSMCs from the posterior tibial artery, harvested from 10 patients with CKD who underwent below-knee amputation due to CLI. Using 1 normal human aortic VSMCs as the control group, we cultured cells in two conditions: normal and high phosphate media. After 10 days of culture, immunofluorescence staining was done. We compared the calcification levels between the two groups using various

assays, tests for cell viability, and scanning electron microscopy. Total RNA was extracted from 9 samples and analyzed through mRNA sequencing and bioinformatics analysis of the DEGs.

Results: Calcification of pathologic peripheral VSMCs increased significantly with time (p=0.028) and was significantly higher than that in normal human aortic VSMCs in calcium assays (p=0.043). Dead cells in the pathologic VSMC group were more distinct in high phosphate media than in normal human aortic VSMCs in the same media. Peripheral VSMCs grown in the calcification media showed a slight decrease after the 7th day of culture. In RNA sequencing, SERTAD4 and ITGA11 were overexpressed in all samples under calcifying conditions by more than 2-fold. The other 7 genes (UNC5B, SPRN, IGFBP6, BCL2A1, APOE, TRABD2A, and FAM13B) showed significant differences with 1.5-fold changes.

Conclusions: VSMCs from the peripheral artery of patients with severe CKD and CLI who underwent amputation surgery showed marked calcifying characteristics compared to normal human aortic VSMCs. A total of 9 genes (SERTAD4, ITGA11, UNC5B, SPRN, IGFBP6, BCL2A1, APOE, TRABD2A, and FAM13B) were found as the suspects related to peripheral vascular calcification.

Keywords: vascular smooth muscle cell; peripheral artery; transcriptome; vascular calcification; chronic kidney disease

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Chapter 1. Introduction

Bone contains 99% of the total calcium in human body. Also called extraskeletal calcification, calcium is also found in various tissues other than bone in several forms, such as hydroxyapatite or amorphous calcium phosphate.[1] Vascular calcification mainly comprises of atherosclerotic calcification within neointimal plaques and medial calcification within the vascular smooth muscle layer.

Both forms of vascular calcification have been studied over decades, due to their close relationship to cardiovascular morbidity and mortality.[1] Atherosclerotic calcification in the intimal layers of the vessel interferes with normal blood flow, and can lead to myocardial/cerebral ischemia, infarction, and even sudden death.[1] Meanwhile, calcific thickening and deposition in the medial layer, also known as Mönckeberg's calcification or medial calcinosis, can increase pulse wave velocity, and may cause systolic hypertension.[1]

Vascular smooth muscle cells (VSMCs) are known to play active roles in the formation of vascular calcification, with various possible mechanisms suggested in previous literatures.[1-3] Vascular calcification is not a singular process; rather, it is a complex pathological process by the VSMCs. Each process may differ according to their histological location in the blood vessel and anatomical location in the arterial tree.[2]

A recent study has demonstrated that the biology of arterial beds differs according to different anatomical sites, leading to different involvement of

VSMCs in vascular calcification.[4, 5] For instance, a thicker fibrous cap, more calcified nodules, and intraplaque hemorrhage are characteristics of vulnerable carotid plaques, compared to coronary plaques.[6] One study suggested that different peripheral vascular beds had heterogeneous nature of arteries, showing distinct gene expression profiles associated with each territory in atherosclerotic and healthy arteries.[7]

Vascular calcification is observed more in patients with chronic kidney disease (CKD), which affects the kidney structure and function..[8] Large observational studies have shown the relationship between vascular calcification and CKD.[2] In response to high extracellular phosphate in CKD, dysregulation of VSMCs is known to facilitate vascular calcification.[2] However, there has been no successful attempt to effectively prevent vascular calcification in patients with CKD.[9] Although some experimental targets involved in calcium deposition were suggested, no intervention has been described to reliably reverse vascular calcification.[9]

Meanwhile, peripheral artery disease (PAD) is caused by insufficient blood flow to the limbs due to the occlusion or narrowing of peripheral arteries. CKD is a significant risk factor for PAD, and the prevalence of PAD is higher in patients with CKD by up to 37%.[10] Patients with PAD often show symptoms such as ischemic pain, claudication, and ulcers. PAD may lead to debilitating conditions such as critical limb ischemia (CLI), often leading to amputation.[11] Current options for the treatment of PAD are limited modalities

such as vascular surgery or balloon angioplasty. Vascular calcification acts as a potential driver of PAD.[12-15] It also has been demonstrated to be a risk factor for the re-stenosis of endovascular stent therapy and increases the risk of limb amputation.[16]

However, previous research regarding the role of VSMCs has been mainly based on major arteries rather than on peripheral arteries. PAD is clinically less significant compared to diseases of major vessels, such as coronary artery disease.[4, 17] Vascular calcification in carotid arteries has also been studied, focusing on characterization of carotid atherosclerotic VSMCs during phenotypic transition.[4] On the other hand, studies regarding PAD have focused mostly on the clinical diagnosis or the treatment, not on its biological characteristics.[18] Scoring systems related to increased severity and complexity of PAD, or histopathologic characterization of peripheral arteries have been developed in the previous literatures.[19, 20] Surprisingly, only a few number of studies focus on the calcifying characteristics of peripheral VSMCs in CKD with CLI.[21]

Understanding the molecular and cellular processes that accelerate vascular calcification may facilitate the development of preventive pharmacotherapy on both cardiovascular disease and PAD.[22] However, a majority of literatures used previously known ontologies (both contractile and synthetic phenotypes) in their comprehensive transcriptomic analyses. Little is known about the pathways related to vascular calcification of the other

human transcriptomes.[4] We believe that exploration of the other genes, which may aggravate or prevent peripheral vascular calcification in the pathologic states, could be the key to prevent peripheral vascular calcification using medical modalities, such as gene therapy.

Thus, we sought to identify the calcifying characteristics of normal VSMCs from major arteries, as well as peripheral VSMCs from patients with CLI, exposed to a high phosphate-concentrated media compared to the conventional media. Additionally, the present study reported a panel of candidate genes, which may contribute to the acceleration of vascular calcification in patients with CLI. We hypothesized that 1) there are some similarities between two kinds of VSMCs under *in vitro* calcifying conditions, and 2) there are some genes or gene sets that participate in the pathologic process of peripheral vascular calcification in patients with CKD, regardless of contractile or synthetic phenotype of VSMCs.

Chapter 2. Materials and Methods

This study was approved by the Institutional Review Board of the Seoul National University Hospital and Myongji Hospital, Hanyang University College of Medicine. Informed consent was obtained from all subjects who participated in this study, which was performed in agreement with the principles outlined in the Declaration of Helsinki.

Patients who met the following criteria were included in the study: 1) patients with CLI who showed necrosis of the foot and required below knee amputations for severe PAD (**Figure 1**); 2) patients with CKD who had an estimated glomerular filtration rate < 60ml/min/1.73m^2; and 3) patients with vascular calcification in the lower leg diagnosed by simple radiographs. We excluded patients who had 1) a history of medial ankle injury of the amputated limb, or had 2) comorbidities that may affect the outcome of vascular biopsy, such as vasculitis.

Final samples were obtained from 10 patients (n=10) who underwent below knee amputations at Seoul National University Hospital (n=8) or Myongji Hospital (n=2).

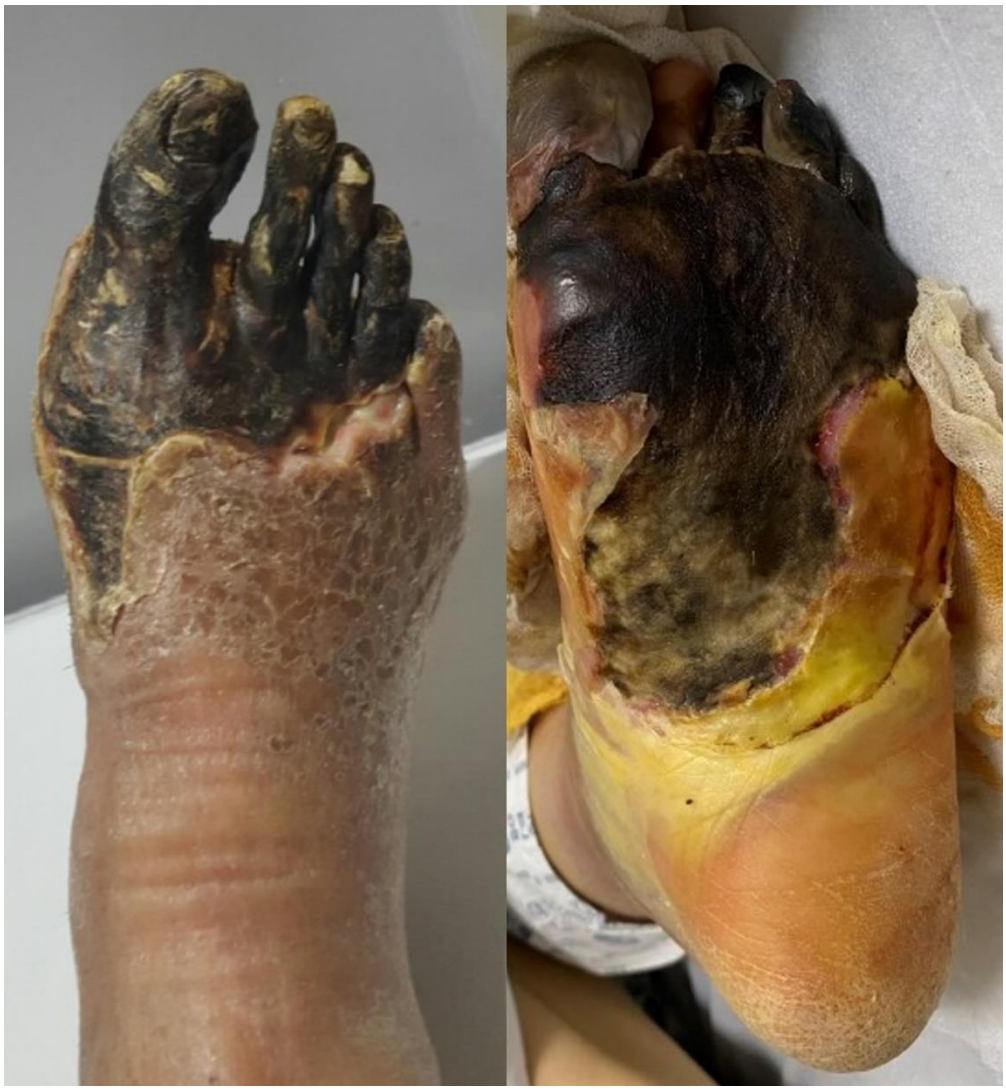


Figure 1. Patient with peripheral artery disease and severe critical limb ischemia, requiring below knee amputation surgery.

Histology of the pathologic vessels

After a macroscopic examination, all pathologic vascular specimens were prepared in an undecalcified manner for embedding using the regular paraffin method, followed by embedding in plastic. Subsequently, the embedded samples were cut to obtain serial sections to prevent artificial damage.[23] The hematoxylin and eosin, and Goldner Masson's trichrome stains were used for the light microscopy analysis.[24]

Isolation and culture of human VSMCs

Vascular biopsies showing pathologies in the amputated legs of patients were obtained at the time of surgery. For isolation of pathologic peripheral VSMCs, we excised about 5 cm of the posterior tibial artery from the amputated ankle. Isolation and culture of the VSMCs were performed by the enzymatic tissue digestion method.[4]

Human vascular aortic smooth muscle cells (catalog no. C-12533) were purchased from PromoCell (Heidelberg, Germany) or isolated from human aorta, using an in-house protocol: the arterial tissues were removed of the adventitia by scraping the endothelium. Arterial strips were washed thrice with 1x phosphate-buffered saline to remove blood, followed by digestion using 2 mg/mL of collagenase type II enzyme (Sigma-Aldrich) for 1 h at 37°C in Dulbecco's modified Eagle medium (DMEM; Gibco, NY, USA). After centrifuge, the pelleted VSMCs were collected and seeded in 6-well plates

containing smooth muscle cell growth medium (SMC-GM2; PromoCell, Heidelberg, Germany) and 1% antibiotic-antimycotic (Gibco). The seeded cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. VSMCs were propagated during the subsequent passages in SMC-GM2, and had an average incubation time of 7 days. Cell viability was assessed using trypan blue after each passage. All assays were conducted with subconfluent cells from passages 1 to 3.[25] For the calcification experiments, VSMCs from both groups were grown using different media depending on the experiment; for the control group, smooth muscle cell growth medium was used, and inorganic phosphate (Pi) concentrations were adjusted using stocks of 3 mM Na_2HPO_4/NaH_2PO_4 (pH 7.4).[25, 26] Medium was changed every two days in all cultures.

Immunofluorescence staining

We performed immunofluorescence staining without cell sorting.[27] Cells were seeded in 6-well plates and grown to 50%–70% confluence. Next, the confluent cells were washed twice with cold 1x phosphate-buffered saline and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Purification of VSMCs was confirmed by immunohistological staining with monoclonal rabbit anti-alpha smooth muscle actin antibody (Abcam, Cambridge, UK), and nuclear staining was performed with the monoclonal mouse anti-transgelin antibody (Santa Cruz Biotechnology Inc., Santa Cruz,

CA) and Hoechst 33258 (Invitrogen, Carlsbad, CA, USA).[28, 29]

The visualization of the calcified deposits

The outlook, microstructure, and crystal structure of the calcium deposits were characterized by field emission scanning electron microscopy (FE-SEM; S-4700; Hitachi, Japan).[30, 31] Samples for SEM observations were prepared by fixing with 2.5% glutaraldehyde, and coating them with gold or carbon. The samples were scanned on a FE-SEM in a range of 10–20 KV.[30]

Calcification assays

To visualize the degree of calcification, we seeded cells in 24-well plates and cultured to 70%–80% confluence. Cells were treated with 3mM Pi and then incubated for 1,3,5,7 and 10 days.[32, 33] Briefly, cells were a fixed with 4% paraformaldehyde for 10 minutes at room temperature, and the cells was stained for 10 minutes with 40 mM Alizarin Red S (pH 4.2; Sigma-Aldrich) at room temperature. The calcium deposits were quantified using a colorimetric kit from BioAssay Systems (Hayward, CA) after solubilization of the deposits for 4 hours at room temperature with 0.6 N HCl.[34] The quantity of calcium deposits were expressed per microgram per milliliter. The deposition of calcium was quantified in a separate set of identically treated cells that were decalcified with HCl after experimentation. Calcium was assayed using the QuantiChrom calcium assay kit (BioAssay Systems,

Hayward, CA, USA) according to the manufacturer's recommendations.[35, 36]

Cell-viability assay

Cell viability was analyzed using a live/dead cell imaging kit (LIVE/DEAD™ Cell Imaging Kit R37601, ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. Cells were seeded in 24-well plates and cultured for 24 hours. Cells were prepared in 24-well plates overnight, and 70%-80% confluent cells were treated with or without 3mM Pi and incubated for 10 days. After Pi treatment, live/dead assay working solution was added to each well, and maintained at room temperature for 15 minutes. Subsequently, cells were observed using a fluorescence microscope **(**DMi8**,** Leica**,** Hesse**,** Germany**),** and green and red fluorescence signals indicated the presence of viable cells and dead cells, respectively. [35, 37]

Next-generation sequencing

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA-Sequencing were carried out by EBIOGEN Inc. (Seoul, South Korea). Briefly, RNA quality control was measured using an Agilent 2100 bioanalyzer with an Agilent RNA 6000 Nano kit (Agilent Technologies, Amstelveen, The Netherlands). RNA quantification was determined using an ND-2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE,

USA). The libraries were constructed using the QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen, Vienna, Austria). High-throughput sequencing was performed as single-end 75 sequencing using a NextSeq 500 (Illumina,Inc., San Diego, CA, USA).

Identification of differentially expressed genes (DEGs)

Analysis of differentially expressed genes and gene ontologies was carried out using the Excel-based Differentially Expressed Gene Analysis (ExDEGA) software program (EBIOGEN, Seoul, Korea).[38-40]

Before screening candidate genes which may be involved in pathologic process of vascular calcification, we identified expression levels of previously known genes related to contractile and synthetic phenotype from all samples in patients with CLI which related to CKD.[4] Primers for genes related with contractile phenotype were α–smooth muscle actin (ACTA2), h-caldesmon (CALD1), calponin 1 (CNN1), and transgelin (TAGLN); for synthetic phenotype were myosin, intercellular adhesion molecule 1 (ICAM1), secreted phosphoprotein 1 (SPP1, also osteopontin), matrix metallopeptidase (MMP) 3, tissue inhibitor of metallopeptidase 1 (TIMP1).

Then, candidate genes were explored from samples using tools described above. Genes expressed with greater than 2-fold change cultured in high phosphate media compared to cells cultured in conventional media. Genes with a p-value<0.05 were considered to be DEGs.[38] We additionally assessed

genes which were significantly upregulated or downregulated by more than 1.5-fold.[41]

Bioinformatics analysis

Scatter plot analysis, heatmap generation/clustering analysis, and gene plot analyses for selected genes was performed using ExDEGA. Ontology sets (from c5.all.v7.4.symbols.gmt) were generated using gene set enrichment analysis (GSEA) 4.1.0 program.[42] The false discovery rate (FDR) was set as $q<0.05$.[43]

Statistical analysis

The quantified data are presented as mean ± standard deviation. Each experiment was replicated three times. The Wilcoxon test was used to compare the quantity of calcification between the 1st and the 10th day. The Mann-Whitney test was performed for comparison between two independent groups at the final day of culture in calcium assay.

Statistical significance was assumed at the 95% confidence level ($p<0.05$). All statistical analyses were conducted using SPSS version 19.0 (IBM Corp., Armonk, NY, USA).

Chapter 3. Results

Mean age of patients was 71.4 years old (56 to 86) and all patients were

male. Six had diabetes, 4 had cardiac vessel disease, 1 had chronic liver disease, and 1 had cerebral infarction history.

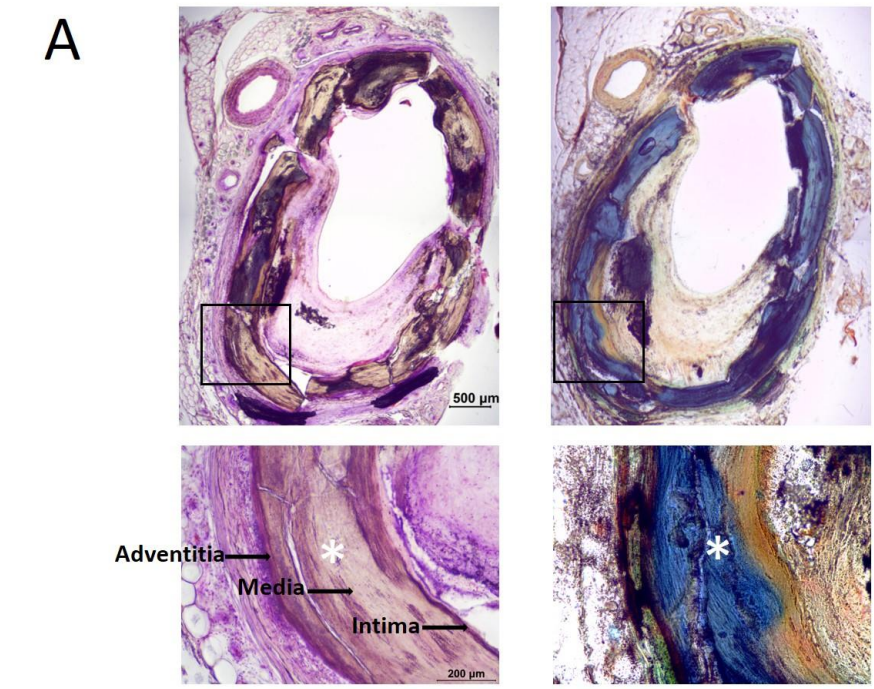
Histology of the pathologic vessels & Immunofluorescence staining

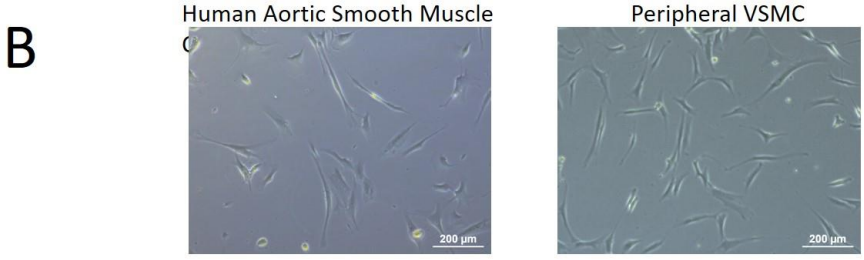
The posterior tibial arteries harvested showed variable phases in the course of the disease, from partially occluded to grossly non-specific. Hematoxylin and eosin stain and Goldner Masson's trichrome stain revealed diffuse and thick deposits (blue-green color) in the tunica media of the undecalcified samples of peripheral arteries harvested from the posterior tibial artery of patients (**Figure 2A**).

After 10 days of culture, we observed VSMCs with elongated and triangular (scarce) morphology in both groups (**Figure 2B**). We confirmed that cultured cells were, in fact, the targeted VSMCs by immunofluorescence staining for SM22α and α-SMA (**Figure 2C**).

The visualization of calcified deposits

On the first day of cell culture under high phosphate conditions, we observed VSMCs clearly in both groups using scanning electron microscope (**Figure 3A**). With time, most cells died, and cell debris with calcium accumulated in the calcifying media (**Figure 3**).


A
500 µm
Adventitia
Media
Intima
200 µm


B
Human Aortic Smooth Muscle
Peripheral VSMC
200 µm
200 µm

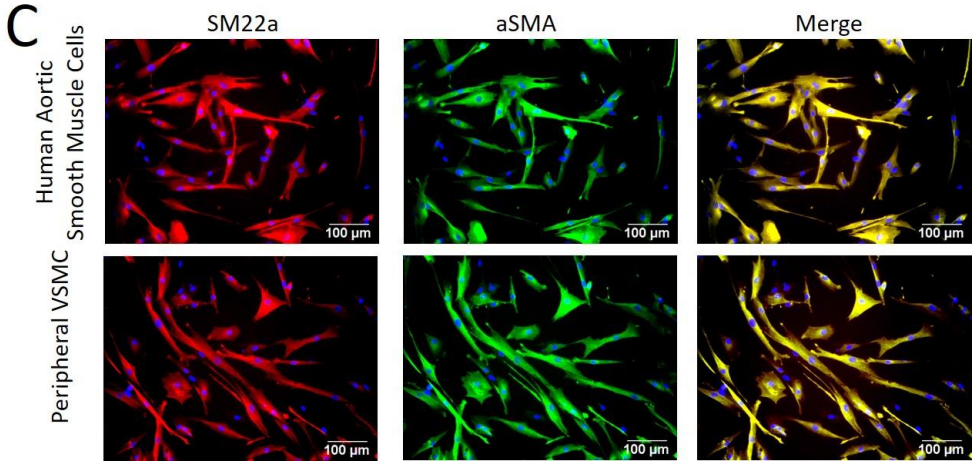

C
SM22a
aSMA
Merge
Human Aortic Smooth Muscle Cells
Peripheral VSMC
100 µm

Figure 2. (A) Hematoxylin and eosin stain showed dense deposits (marked as asterisks) in tunica media. Calcified matrix (blue-green color) was observed in Goldner Masson's trichrome stains. (B) After 10 days of culture in the general media, elongated or triangular shaped VSMCs were observed. (C) We confirmed that VSMCs were cultured through immunofluorescence staining using SM22α and α-SMA.

Calcification assays

We examined calcification in pathologic VSMCs and normal human aortic VSMCs using Alizarin Red S staining. Calcification was more prominent and significantly higher in the pathologic VSMCs from peripheral arterial cells than in normal human aortic VSMCs, when grown in calcification media ($p=0.043$) (**Figure 4A, 4B**). Calcification of pathologic VSMCs after 10 days of culture in the calcification media was significantly elevated compared to Day 1 ($p=0.028$), and showed a significant difference between cells grown in the calcification media relative to the general media after 10 days of culture ($p<0.001$) (**Figure 4C**). In contrast, calcification of pathologic VSMCs and normal human aortic VSMCs grown in the general media showed no differences after 10 days of culture ($p=0.735$) (**Figure 4C**).

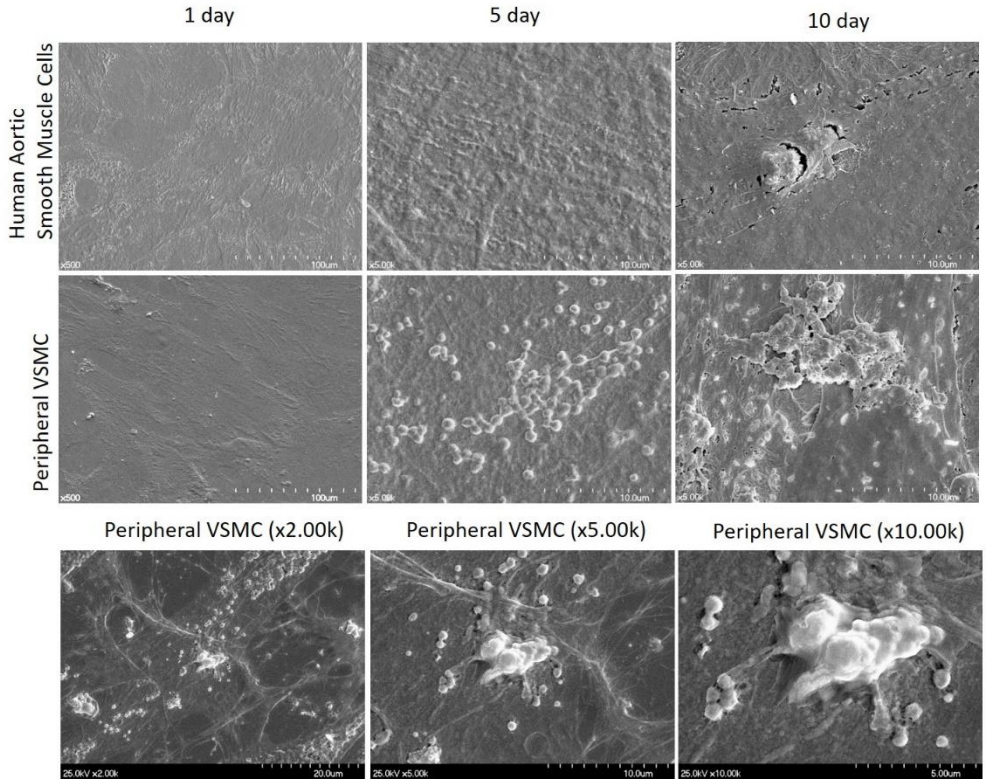


Figure 3. Scanning Electron Microscope of normal human aortic vascular smooth muscle cells and pathologic VSMCs (vascular smooth muscle cell) in calcifying media obtained with a magnification of 5000 at 1KeV.

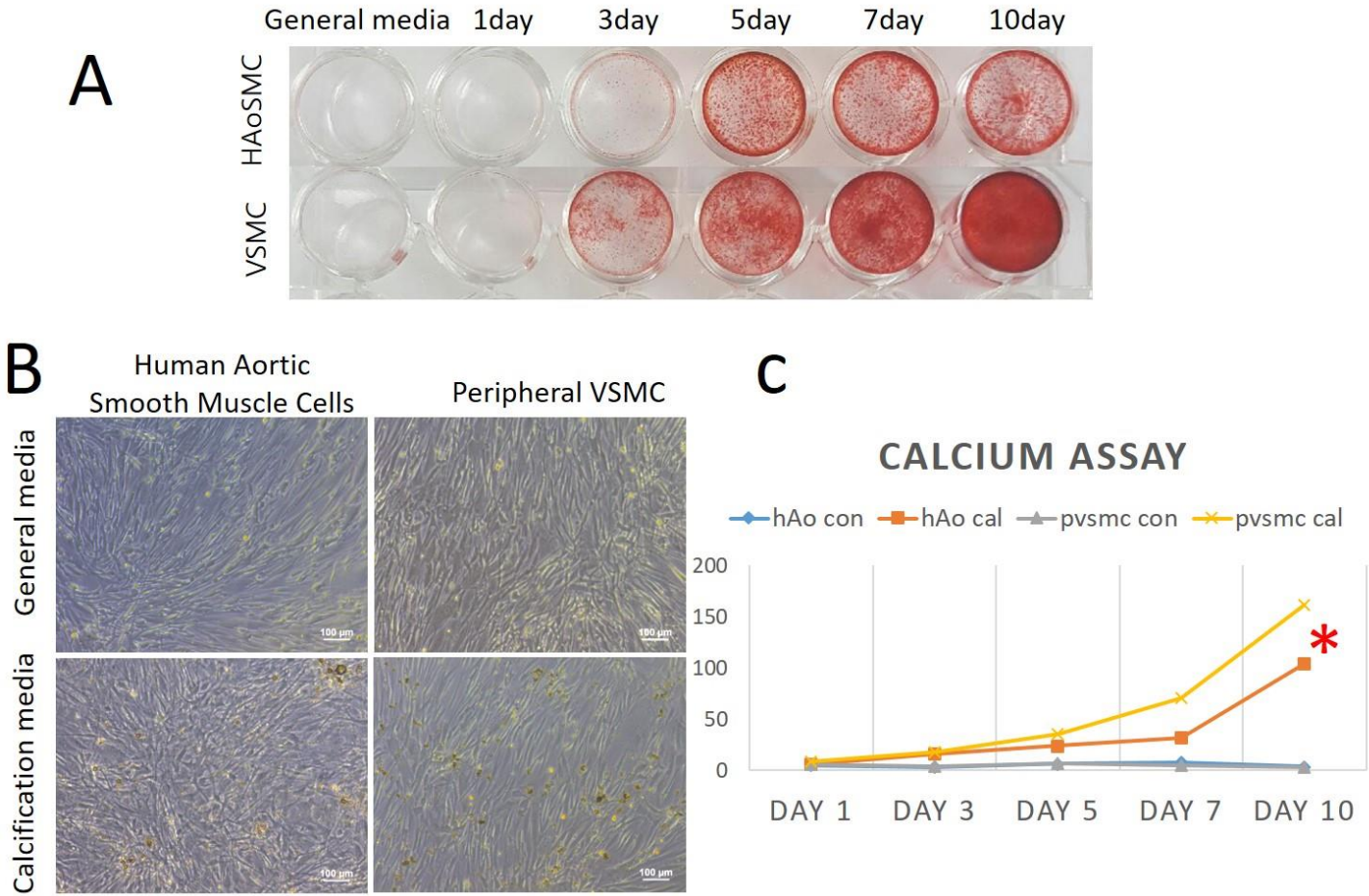

A
General media
1day
3day
5day
7day
10day
HAoSMC
VSMC
B
Human Aortic
Smooth Muscle Cells
Peripheral VSMC
General media
Calcification media
100 µm
C
CALCIUM ASSAY
hAo con
hAo cal
pvsmc con
pvsmc cal
200
150
100
50
0
DAY 1
DAY 3
DAY 5
DAY 7
DAY 10
*

Figure 4. Calcium assay and quantitative analysis. (A) Alizarin Red S staining showed outstanding red stains in pathologic VSMC (vascular smooth muscle cell) compared to normal HAoSMC (human aortic smooth muscle cell) in the calcification media. (B) Specific shapes of VSMCs, observed in the general media, were lost in the calcification media. Calcium deposit in the calcification media was more prominent in the peripheral VSMC group. (C) Pathologic VSMCs in the calcification media showed most calcification among four groups, followed by human aortic VSMC in the calcification media. Asterisk indicates significant difference of calcification between pathologic VSMC and human aortic VSMC in the calcification media. Calcification of pathologic VSMC and human aortic VSMC in the general media was minimal.

hAo (human aortic smooth muscle cell), psvmc (peripheral vascular smooth muscle cell), con (in general media as a control group), and cal (in calcification media)

Cell-viability assay

Dead cells, shown as red dots, of the pathologic VSMC group in the calcification media considerably increased, compared to the other 3 groups (**Figure 5A**). The number of viable VSMCs from peripheral arterial cells grown in calcification media showed a slight decrease after 7 days of culture (**Figure 5B**).

DEG identification

Excluding 2 samples that showed inconsistent outcomes in two repetitive sessions of RNA sequencing, 8 pathologic samples were included in the analysis. Interestingly, all 4 genes related to the contractility of VSMCs (ACTA2, CALD1, CNN1, and TAGLN) were significantly upregulated by more than 1.5-fold, only in normal human aortic VSMC in calcifying media; no significant change or significant downregulation was seen in pathologic peripheral VSMCs. Four genes related to synthetic phenotype (ICAM1, SPP1, MMP3, and TIMP1) showed no significant change or significant downregulation in all samples.

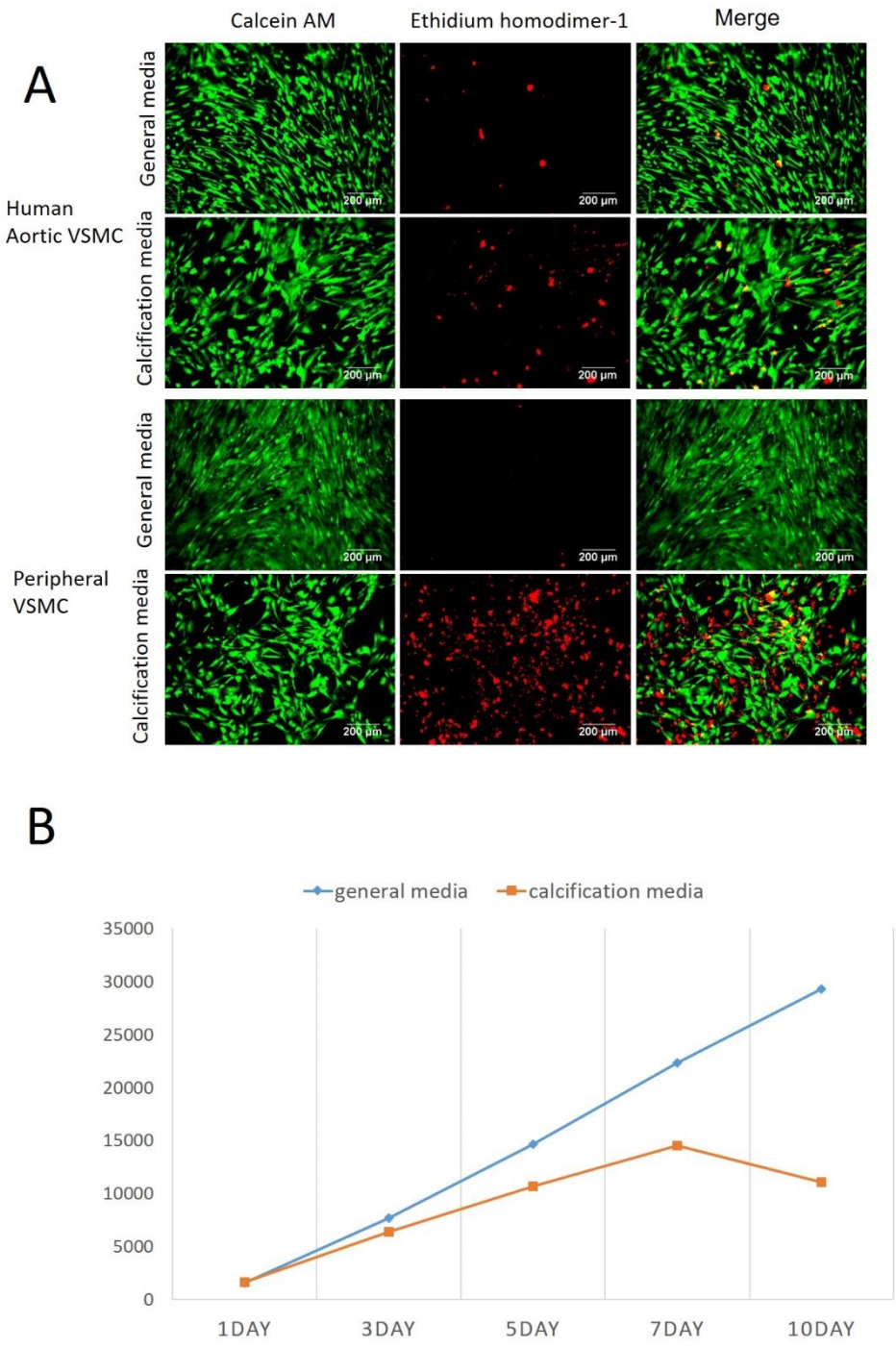


Figure 5. Viability of pathologic VSMC and human aortic VSMC in both normal media and high phosphate media. (A) They were incubated with

calcein-AM(acetoxymethyl ester), EthD-1, and annexin V-AlexaFluor® 647 conjugate (Life Technologies, Carlsbad, CA, USA) to label live cells (green), and dead cells (red). (B) The number of living VSMCs from peripheral arterial cells cultured in the calcification media slightly decreased after 7 days.

Then, we analyzed the whole transcriptome RNA sequencing to identify the differences of the gene expression profiles between the calcifying media and the normal media. Scatter plot analysis showed that the DEG profiles of samples overlapped considerably (**Figure 6**).

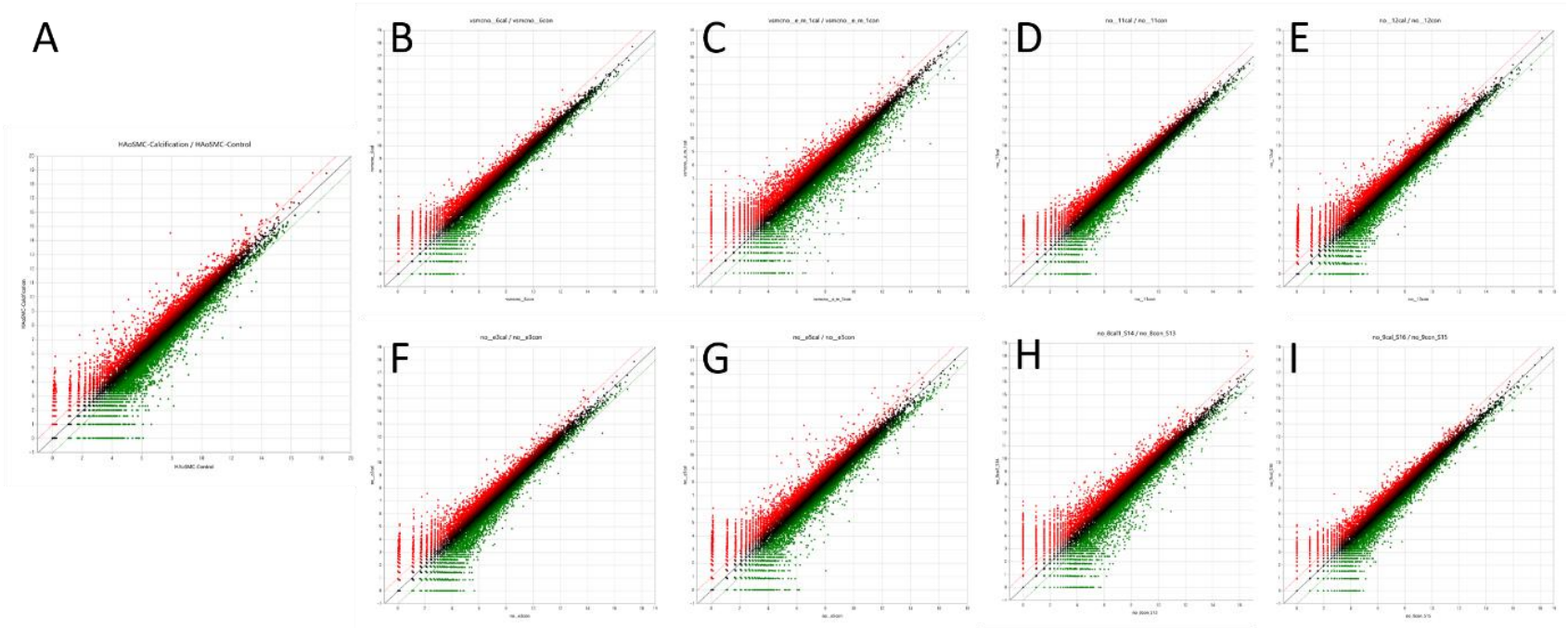


Figure 6. Scatter plots of gene expression. (A) Expression in normal human aortic VSMC as the control group. (B) - (I) Expression in each pathologic peripheral VSMCs. In each plot, the central line passing through the origin indicates no difference in expression between the cells stored for different time periods. Red plots indicate up-regulated genes, and green plots present down-regulated genes. Plots outside the dotted line (red or green) indicate more than a 2-fold difference.

Heatmap generation and clustering analysis

Comparing expression differences of cells exposed to the calcifying media respect to the normal media, the generated heatmap shows that normal human aortic VSMCs respond similarly to the pathologic peripheral VSMCs (**Figure 7**).

Gene plot analysis

We detected only 2 genes, SERTAD4 and ITGA11, showing up-regulation by more than 2-fold change in the calcifying media, compared to the cells cultured in general media. These two genes were overexpressed in all samples, including normal human aortic VSMC, which was the control group.

Seventeen genes showed more than 1.5-fold change but less than 2-fold change. Ten genes were excluded because they showed conflicting patterns in expression; some samples showed over-expression, and others showed under-expression at the same time. After exclusion of 10 genes, we found 7 genes (UNC5B, SPRN, IGFBP6, BCL2A1, APOE, TRABD2A, and FAM13B) showing consistent patterns in all pathologic peripheral VSMC samples. Among them, UNC5B was overexpressed in all pathologic peripheral VSMC samples, while showing under-expression in the control group. Expression of 5 genes (SPRN, IGFBP6, BCL2A1, APOE, TRABD2A)

were decreased in all samples including the control group. FAM13B was overexpressed in all samples.

Expression of selected 9 genes in response to the calcifying media compared to the general media is shown (p-value<0.05) (**Figure 8**).

Gene Set Enrichment Analysis (GSEA)

The 5 significant enriched gene sets from samples cultured in general media as the control group were detected; gene ontology of cytosolic large ribosomal subunit, cytosolic ribosome, cotranslational protein targeting to membrane, protein localization to endoplasmic reticulum, and structural constituent of ribosome (**Figure 9**). No gene sets were enriched in samples cultured in calcifying media.

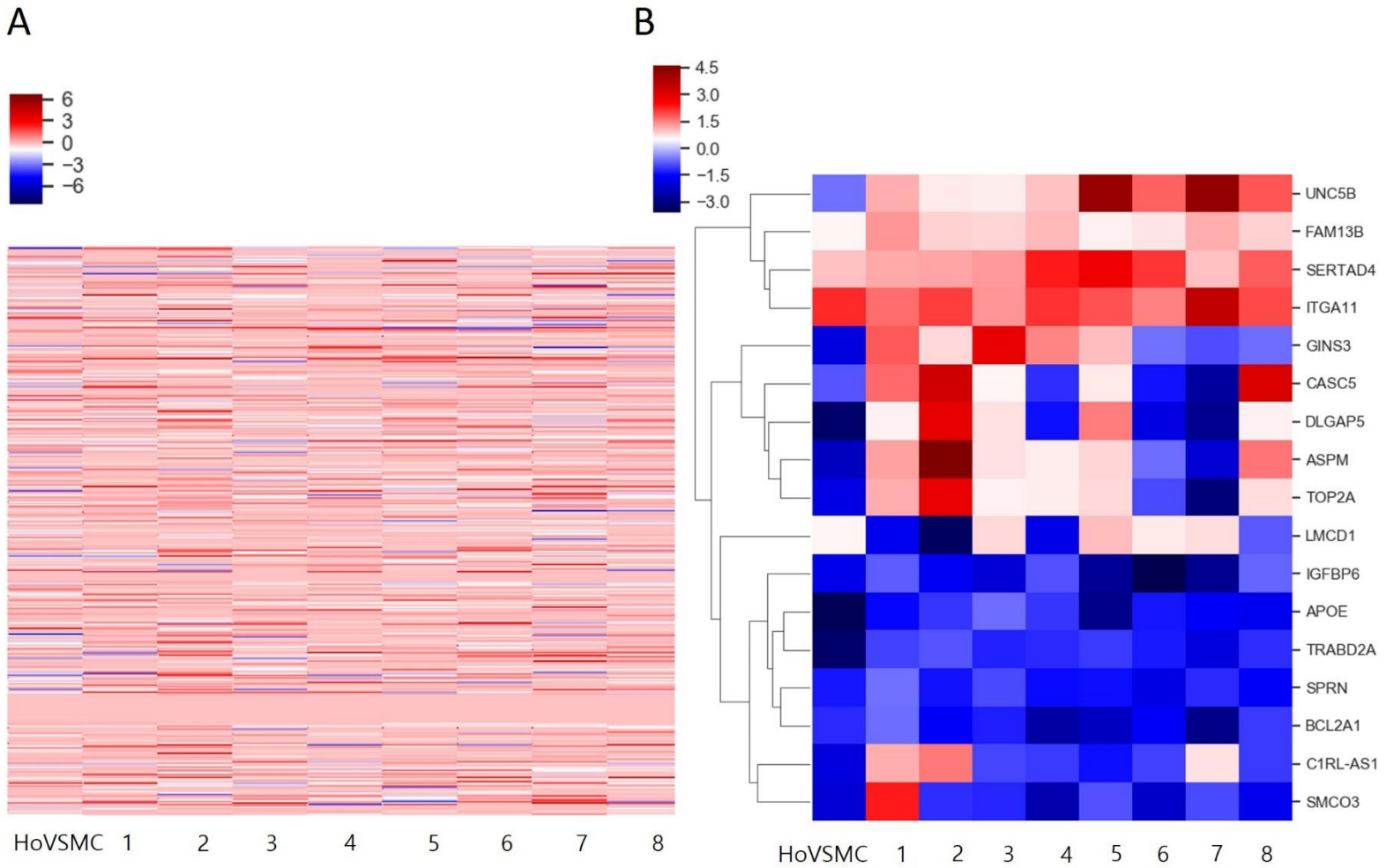

A
6
3
0
−3
−6
HoVSMC 1 2 3 4 5 6 7 8
B
4.5
3.0
1.5
0.0
−1.5
−3.0
UNC5B
FAM13B
SERTAD4
ITGA11
GINS3
CASC5
DLGAP5
ASPM
TOP2A
LMCD1
IGFBP6
APOE
TRABD2A
SPRN
BCL2A1
C1RL-AS1
SMCO3
HoVSMC 1 2 3 4 5 6 7 8

Figure 7. (A) Heat map and hierarchical clustering of the RNA sequencing data from all samples. (B) Clustered heatmap showing expression differences of cells exposed to the calcifying media respect to the normal media. Numbers 1 to 8 indicate samples of the pathologic peripheral VSMCs, and HoVSMC indicates human aortic VSMC, the control group.

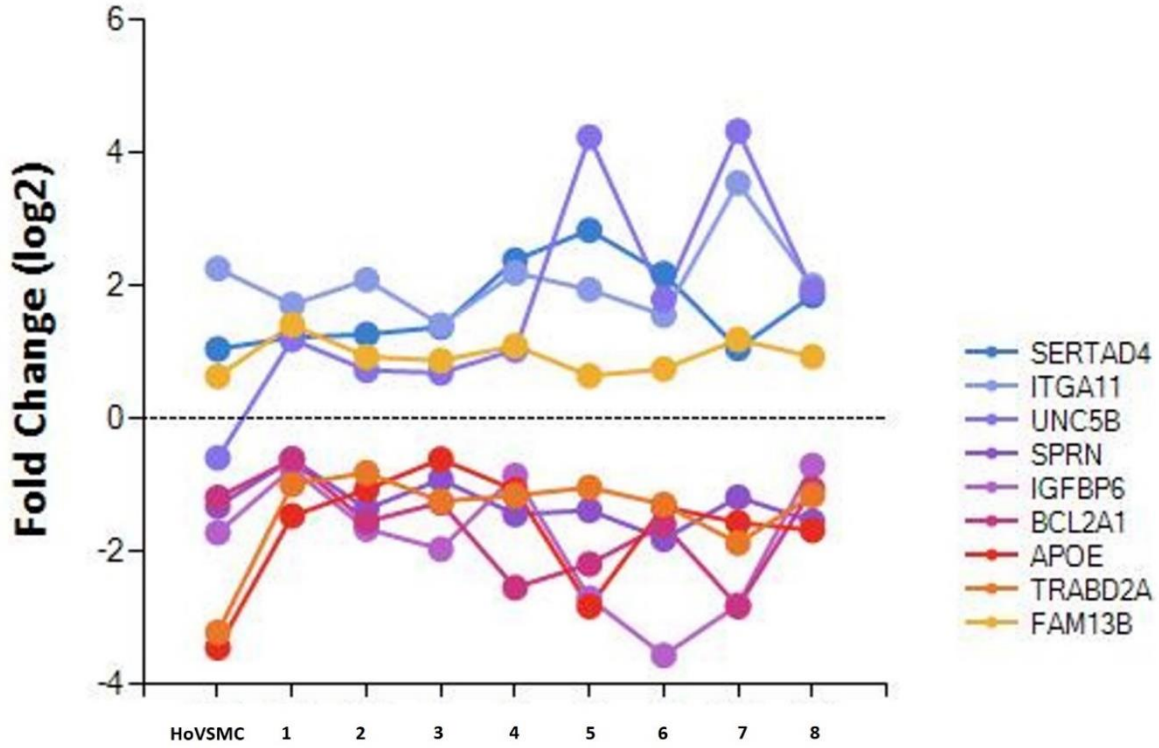


Figure 8. Gene plot showing expression of selected 9 genes. Note that graphs above the value 0 (shown as dotted line) indicate up-regulation, and graphs below 0 indicate down-regulation.

A

Gene set details	ES	NES	NOM p-val	FDR q-val
GOCC_CYTOSOLIC_LARGE_RIBOSOMAL_SUBUNIT	55	0.73	2.22	0.000
GOCC_CYTOSOLIC_RIBOSOME	104	0.63	2.09	0.000
GOBP_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	102	0.59	1.98	0.000
GOBP_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION_TO_ENDOPLASMIC_RETICULUM	116	0.58	1.98	0.000
GOMF_STRUCTURAL_CONSTITUENT_OF_RIBOSOME	160	0.55	1.93	0.000

B

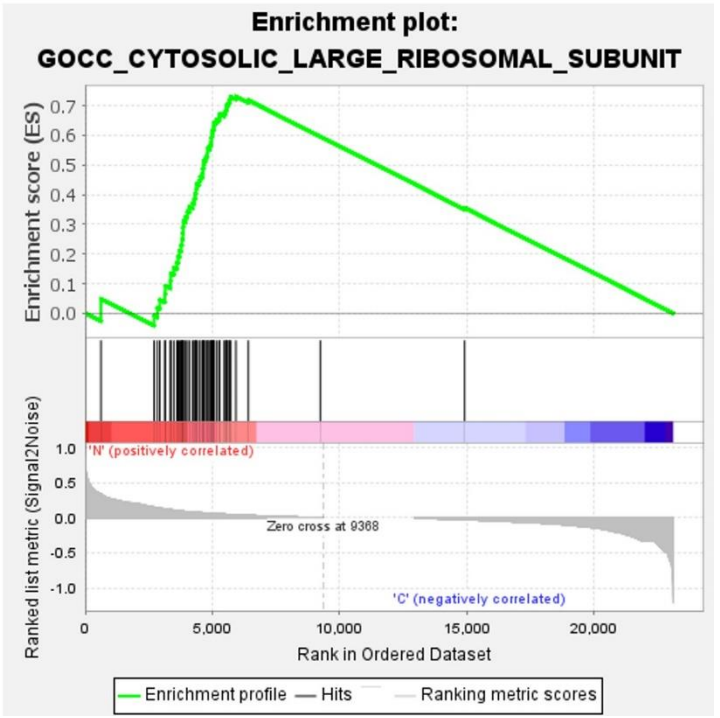


Figure 9. Gene set enrichment analysis. (A) Only gene sets with q-value<0.05 were included, all of which were from samples cultured in general media. No significant gene sets from samples cultured in calcifying media were screened in the c5 gene ontology set analysis. (B) One of 5 gene sets enrichment plots with significant q-value from samples cultured in general media.

Chapter 4. Discussion

This study reports the following for the first time: 1) calcifying characteristics of pathologic peripheral VSMCs, and 2) nine candidate genes related to peripheral vascular calcification in patients with CKD and CLI, using RNA sequencing. Prominent medial wall calcification was observed in samples harvested from the posterior tibial artery of CLI patients with CKD. VSMCs from the pathologic peripheral artery showed pro-calcifying characteristics relative to normal human aortic VSMCs, even after several culture passages. Further, the viability of pathologic VSMCs from the peripheral artery was poorer than that of normal aortic VSMCs when grown in calcification media.

Using truly calcified posterior tibia arteries harvested from patients with CKD and CLI who underwent below knee amputation, this study possesses novelty as most studies in previous literatures have used aortic arteries and simulated vascular calcification *in vitro*.

In vitro and *in vivo* studies have shown that VSMCs play an active role in generating osteochondrogenic precursors by transdifferentiation in calcifying arteries.[44] A majority of previous clinical studies have focused on vascular calcification in major vessels such as coronary arteries, carotid arteries, and the aorta or its adjacent branches.[45] Few reported studies regarding vascular calcification of the peripheral arteries, especially in patients with CKD and diabetes, have focused on the characteristics of

atherosclerotic plaques in the peripheral arteries. One study reported the plaque characteristics in patients with PAD undergoing primary endarterectomy, which showed a time-dependent shift towards a less lipid-rich lesion and reduced intraplaque hemorrhage.[18] The other study evaluated arterial pathology in the entire length of the lower extremities.[20]

Clinical presentation or calcifying characteristics between major arteries and peripheral arteries may differ by a great magnitude.[46] We revealed that pro-calcifying characteristics of VSMCs in patients with PAD were similar to those of *in vitro* studies regarding VSMCs in major vessels.[11] However, the degree of calcification in pathologic peripheral VSMCs was much more than that in normal aortic VSMCs. Several candidate genes were detected from samples of this study by RNA sequencing and bioinformatics. Expression of two genes, SERTAD4 and ITGA11, was significantly increased in calcifying conditions regardless of normal major or pathologic peripheral VSMCs. UNC5B gene was overexpressed exclusively in the pathologic peripheral VSMCs. Expression of 5 genes (SPRN, IGFBP6, BCL2A1, APOE, TRABD2A) were decreased in all samples, while FAM13B was overexpressed in all samples.

It has been proved that elevated serum phosphate salt is a risk factor for vascular calcification among CKD populations from clinical studies.[47, 48] In fact, calcification of VSMCs can be triggered by high phosphate treatment; however, the exact mechanism remains unclear.[47] High phosphate-inducing

method for calcification of VSMCs is used to discover the actual underlying mechanism.[49] We also used this method to observe the calcifying characteristics of peripheral VSMCs harvested from CKD patients.

It is well known that VSMCs in pathological conditions can dedifferentiate into a synthetic phenotype, and facilitate vascular calcification. There are many studies exploring various pathways involved.[50] This study focused on comparison of behavior of VSMCs in response to the calcifying media with respect to the normal media, as well as between two kinds of cells (diseased peripheral VSMCs and normal major VSMCs) under the same conditions. Interestingly, cells cultured from diseased peripheral VSMCs showed greater calcification compared to those from normal major VSMCs, while both groups had similar common features of VSMCs. Such similarity was confirmed by microscopic cell morphology and immunofluorescent staining. Cells cultured from patients' peripheral VSMCs may have synthetic phenotypes, which may have led to such results. Future studies would look into which phenotypes the cells from pathologic peripheral VSMC may have activated.

Under various stressful conditions, VSMCs have three main options: autophagy, senescence, or apoptosis.[51] The choice of each of strategy likely depends on the strength of the stimulus, the time of exposure, and the state of the VSMCs.[51] Previous studies have suggested that high phosphate conditions promote vascular calcification, stimulate osteogenic or chondrogenic

differentiation, induce vesicle release, and cause apoptosis.[2, 52, 53] We observed that growth rate of pathologic peripheral VSMCs under high phosphate media was lower than that under general media. Moreover, the number of viable VSMCs from peripheral arterial cells grown in high phosphate media showed a slight decrease after 7 days of culture, implying increased cell death of grown VSMCs.

Actually, VSMC death was more studied in atherosclerosis than in medial wall calcification.[51] In atherosclerosis, cell death have two major forms: necrosis and apoptosis. Necrosis is characterized by a gain of cell volume and swelling of organelles, while apoptosis is a programmed cell death.[51] Although we observed VSMC death and calcified vesicles on ruptured cell membranes by SEM, we could not differentiate between necrosis and apoptosis. Future studies should elaborate on the causal relationship between VSMC death and calcification.

Two distinct forms of vascular calcification, medial wall calcification within the smooth muscle layer and atherosclerosis, are significant risk factors for cardiovascular events, alteration of bone and mineral metabolism, or death.[9] Vascular calcification, in the lower extremities of patients with CKD and diabetes, is reported to occur mostly in the tunica media. Approximately 20% of patients who underwent the ankle-brachial index test for CLI were found to have non-compressible arteries due to tibial artery calcification.[54] Our results from experiments using the posterior tibia artery

were consistent with these studies as we have identified vast amounts of calcified deposits in the medial wall.

We selected posterior tibia artery as the harvest sample because more distal arteries were affected by forefoot necrosis. Previous studies regarding peripheral artery disease used more proximal arteries, such as the femoral artery. Posterior tibia artery was the most distal artery of all viable peripheral arteries, without severe infection or necrotic changes.

We think that VSMCs cultured in our *in vitro* study mimicked survived VSMCs in the tunica media of severely calcified peripheral arteries. Therefore, it can be implied that peripheral VSMCs from patients with advanced CKD and PAD lack the ability to suppress calcification under pro-calcifying stimuli compared to normal VSMCs, even though they do not show such characteristics under normal circumstances. Therefore, clinicians should keep in mind that vascular calcification of peripheral arteries in CKD patients would be more severe, and may cause various complications.

Before this study, we had expected that expression of genes related to contractility would be decreased and genes related to synthetic phenotype would be overexpressed in all samples under calcifying conditions.[4, 55] However, the result was different from our expectation. Human aortic VSMCs as the control sample showed increased expression of genes related to contractility phenotype, although it was not statistically significant. Meanwhile, expression of genes related to synthetic phenotype of VSMCs

decreased or showed no significant changes in all samples under calcifying conditions. There may be some kind of defensive mechanism, which would be defective in pathologic peripheral VSMCs, against calcifying stimuli in normal aortic VSMCs. Functions of specific genes that we detected in this study have not been fully elucidated in previous literatures. Proteins containing the SERTAD4 domain have previously been linked to cell cycle progression and chromatin remodeling.[56] SERTAD4 is known to be expressed in many kind of tissues, such as the smooth muscles in the heart, smooth muscles, and the skeletal muscle. One study suggested that SERTAD4 was one of 1431 upregulated genes in inhibition of atherosclerosis development.[57]

ITGA11, integrin alpha 11, is mainly expressed in female genital organs and smooth muscles. Bansal et al. reported that ITGA11 was co-localized with α-smooth muscle actin-positive myfibroblasts and was induced with increasing fibrogenesis in human fibrotic organs.[58] ITGA11 knockdown dramatically altered the myofibroblast phenotype, including impaired contractility of collagen I matrices in their study.[58] Our findings support their study which highlight the significance of the ITGA11 receptor as a promising therapeutic target in organ fibrosis and vascular calcification.

Interestingly, we noticed that expression of one gene (UNC5B) showed an opposite pattern between normal human aortic VSMCs and pathologic peripheral VSMCs: under-expression in human aortic VSMC and over-expression in pathologic peripheral VSMCs. One study suggested that netrin-

1 receptor UNC5B plays a critical role in cell survival and kidney injury.[59] Other studies suggested Netrin-1 receptor UNC5B plays important roles in angiogenesis in diabetic kidney disease.[60] We suspect that UNC5B, which was overexpressed in pathologic peripheral VSMCs cultured in the calcifying media, is closely related to PAD.

The other genes (SPRN, IGFBP6, BCL2A1, APOE, TRABD2A, and FAM13B) showed over- or under-expression in all samples under calcifying conditions. SPRN is related to protein coding of diseases such as Crutzfeldt-Jakob disease. IGFBP6 is an insulin-like growth factor-binding protein 6, while BCL2A1, mainly expressed in the hematopoietic system, is highly regulated nuclear factor kB target gene that exerts important pro-survival functions. APOE is related to apolipoprotein E, and TRABD2A is a negative regulator of the Wnt signaling pathway. Further studies would be needed regarding the relationship between peripheral vascular calcification and these genes.

This study has several limitations. First, we did not compare cells from the same origin, which may have biased our results. Analyzing samples from major vessels such as the aorta of the same patient from whom we harvested peripheral vessels could have provided more insights. However, it is not possible to obtain such samples due to ethical issues. Instead, we performed subgroup analysis for a fair comparison of calcifying character of cells; culture of the same specimen of each group in both general media and

calcifying media. Second, the sample size of this study is relatively small as recent medical advances have made limb salvage possible, thus reducing the need for amputation surgery. Third, it would have been better that we had analyzed the whole process of vascular calcification by observing the transformation of normal peripheral VSMCs into pathologic peripheral VSMCs. Our study population included only patients with terminal stage of PAD, who underwent below knee amputation. Moreover, phenotypes of VSMCs cultured through several passages may be different from those of VSMCs in the posterior tibia arteries *in vivo*. We tried to use VSMCs within 3 culture passages to minimize this weakness. Therefore, we think that further studies regarding 1) DEGs identification by animal model using other modalities such as PCR, and 2) exploration of biochemical pathways of the selected genes affecting peripheral vascular calcification are required.

Chapter 5. Conclusion

We observed marked calcifying characteristics in VSMCs from the peripheral arteries of patients with severe CKD and CLI who underwent amputation surgery, when compared to normal human aortic VSMCs. We detected nine genes that may be related to peripheral vascular calcification from DEG analysis. We believe our study provided a basis for identification of the distinct characteristics of peripheral VSMCs related to PAD, and provided valuable information for future therapeutic approaches in PAD.

Further studies are needed to explore the exact role of the candidate genes in peripheral vascular calcification.

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국문 초록

신장 질환으로 인한 말초혈관 질환 환자의 말초 혈관 평활근 세포와 정상 대동맥 평활근 세포의 고인산 환경에서의 석회화 비교 및 RNA sequencing을 통한 발현 전사체 차이 분석

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의학과 정형외과학 전공

목적: 만성 신장 질환에서 흔한 혈관 석회화에서 말초 혈관 평활근 세포의 역할은 대동맥 혈관 평활근 세포에 비해 연구된 바가 거의 없다. 본 연구는 만성 신장 질환 및 말초 동맥 질환 환자에서 말초 혈관 평활근 세포의 석회화 특성을 연구하고, 고인산 배양 환경에서 발현되는 유전자를 도출하고자 하였다.

대상 및 방법: 만성 신장 질환 및 말초 동맥으로 하지 절단한 총 10명의 환자를 대상으로 하였고 대조군으로 상업용 정상 대동맥 혈관 평활근 세포를 사용하였다. 절단지의 후경골동맥에서 말초 혈관 평활근 세포를 추출하고 이를 정상 배지 및 고인산 배지에서 열흘간 배양하여 석회화 정도, 세포 생존력을 비교하고 면역형광염색 및 전자현미경으로

관찰하였다. RNA 염기서열분석과 생물정보학 분석을 통해 정상 환경 대비 고인산 환경에서 유의미하게 발현된 유전자를 도출하였다.

결과: 고인산 환경에서 말초 혈관 평활근 세포의 석회화는 10일 후 유의하게 증가하였으며(p=0.028), 정상 대동맥 혈관 평활근 세포에 비해서도 유의하게 증가하였다(p=0.043). 고인산 환경에서 말초 혈관 평활근 세포의 사멸이 정상 대동맥 혈관 평활근 세포보다 뚜렷하였다. RNA 염기서열분석상 모든 표본에서 SERTAD4 과 ITGA11 는 2배 이상 유의하게 과발현되었으며, 7개의 유전자(UNC5B, SPRN, IGFBP6, BCL2A1, APOE, TRABD2A, and FAM13B) 는 1.5배 이상 유의하게 과발현 혹은 저발현되었다.

결론: 하지 절단에 이르는 만성신장 질환에서의 말초혈관 질환 환자에서 말초 혈관 평활근 세포는 정상 대동맥 혈관 평활근 세포에 비해 뚜렷한 석회화 형질을 가지고 있었다. 총 9개의 유전자 (SERTAD4, ITGA11, UNC5B, SPRN, IGFBP6, BCL2A1, APOE, TRABD2A, and FAM13B) 가 말초 혈관 석회화 현상에 연관있을 것으로 의심하였다.

색인 단어: 혈관 평활근 세포, 말초 혈관, 전사체, 혈관 석회화, 만성 신장 질환

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