Zmpste24 deficiency accelerates atherosclerosis by increasing TLR4 expression through epigenetic modification in vascular smooth muscle cells

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Zmpste24 deficiency accelerates atherosclerosis by increasing TLR4 expression through epigenetic modification in vascular smooth muscle cells

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ABSTRACT

Zmpste24 deficiency accelerates atherosclerosis by increasing TLR4 expression through epigenetic modification in vascular smooth muscle cells

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(Directed by Professor Sungha Park)

<Objective>

Atherosclerosis is a progressive chronic inflammatory disease and is the leading cause of mortality worldwide. Advancing age is a major risk factor for atherosclerotic

cardiovascular disease; however, the mechanisms underlying this phenomenon remain unclear. Therefore, the aim of the present study was to investigate the impact of the vascular aging-related gene, Zmpste24, on vascular smooth muscle cell phenotypic switching and acceleration of atherosclerosis, and elucidated the underlying mechanism.

<Methods>

Vascular smooth muscle cells isolated from whole rat aorta were used for this study. They were treated with Zmpste24 siRNA and cholesterol (20 μg/ml) for 48 hours in serum free-DMEM. Cholesterol accumulation of VSMCs was visualized by oil red O staining. The expression of RNA and protein associated with phenotypic switching and signaling pathways were analyzed by RT-PCR and western blot analysis. In vivo, $Zmpste24^{F/F}$ $SM22\alpha Cre^{+}$ $ApoE^{-/-}$ mice via Tamoxifen IP were fed a high cholesterol diet to induce atherosclerosis over 8 weeks.

<Results>

I confirmed using oil red O staining that cholesterol increased VSMC-foam cell formation in an in vitro model. Cholesterol induced VSMC phenotype switching by downregulating SMC marker expression and upregulating macrophage marker expression. Knockdown of Zmpste24 significantly enhanced VSMC-foam cell formation and cholesterol-VSMC phenotype switching. Conversely, overexpression of Zmpste24 reduced VSMC-foam cell formation. Animal studies revealed that $Zmpste24^{F/F}$ $SM22\alpha Cre^{+}$ $ApoE^{-/-}$ mice accelerated atherosclerosis in the aorta of mice fed with a high cholesterol diet.

Consistent with the in vitro model, cholesterol-VSMC phenotype switching was accelerated in the animal model.

<Conclusion>

The present results suggest that Zmpste24 deficiency accelerates atherosclerosis by effectively increasing cholesterol-induced VSMC-foam cell formation and cholesterol-VSMC phenotype switching. In addition, Zmpste24 deficiency promotes cholesterol accumulation by regulating TLR4 expression through epigenetic modification in VSMCs. Taken together, the results may provide insight for the development of drugs to prevent atherosclerosis protection during cardiovascular disease.

Key words: Atherosclerosis; Zmpste24; TLR4; DNMT3a; Epigenetic modification

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I. INTRODUCTION

Introduction

Atherosclerosis is a chronic inflammation disorder characterized by the deposition and

accumulation of lipid in the arterial intima [1]. Vascular smooth muscle cells (VSMCs) are a major cell type at all stages of an atherosclerotic plaque formation. VSMCs are more plastic than previously recognized and can adopt alternative phenotypes, including phenotypes resembling foam cells and macrophages, which may both positively and negatively contribute to disease progression. During early atherosclerosis, VSMCs are the predominant cell type within the plaque. Uptake of oxidized-LDL by VSMCs results in foam cell formation and apoptosis. Activated VSMCs secrete chemokines and contribute to the recruitment of monocytes, which differentiate into macrophages. Progression to pathological intimal thickening is typically associated with a decreased number of cells with VSMC markers and an increased number of cells with macrophage markers. This likely reflects a combination of VSMC death and VSMC phenotype switching to macrophage-like cells. A previous report suggested that 40% of intimal SMCs contribute to foam cell formation and cholesterol hyper-accumulation [2].

Zinc Metallopeptidase STE24 (Zmpste24) is a gene associated with aging and, as an enzyme, it converts prelamin A into mature lamin A [3]. A lack of Zmpste24 or defects in the Zmpste24 gene cause laminopathies with defects in lamin A and increases DNA damage, nuclear morphology defects, DNA repair system defects, and premature senescence [4] [5]. In addition to these genetic problems, Zmpste24 function is impaired even in normal people because of oxidative stress in blood vessels [6] [7]. Zmpste24 whole-knockout mice exhibit prelamin A accumulation in the nuclear membrane, abnormal nuclear morphology, loss of subcutaneous fat, skeletal and muscular defects, hair loss, and

metabolic alterations that result in shortened lifespan[8] [9]. It has also been shown that Zmpste24 deficiency accelerates aging through activation of p53 signaling [10], and also has been shown to accelerate atherosclerosis [11]. Also, Prelamin A is said to accelerate VSMC aging by inducing mitotic defects and DNA damage [7].

Vascular aging leads to an increase in collagen and fibrosis as well as intimal and medial thickening (vascular remodeling) of blood vessels which, in turn, leads to an increase in vascular stiffness. It causes high blood pressure and promotes arteriosclerosis. In addition, aging blood vessels show increased expression of pro-inflammatory molecules and increased plasma lipoproteins uptake, which is known to contribute to atherosclerosis [12]. Looking at the histological characteristics of aging-related Hutchinson-Gilford progeria syndrome, the extensive loss of VSMC in the medial layer, replacement of VSMC in the intima and media with fibrous tissue [13], the breakdown of VSMC and elastin, and progerin accumulation in the aorta of 12-month-old mice, was confirmed by H&E and Movat's staining of arteries from HGPS-progeria mice [14]. Although aging is known as a major risk factor for atherosclerotic cardiovascular disease from these epidemiological studies, the molecular mechanisms of aging-induced atherosclerosis are not fully understood.

Endothelial cell dysfunction in atherosclerosis has been studied extensively, but relatively little is known regarding the effect of VSMCs on atherosclerosis. However, in recent years, there have been an increasing number of studies on the role of VSMCs in atherosclerosis. Many studies have examined the phenotypic switching of VSMCs. The

phenotypic switching of VSMCs from contractile phenotype to a proliferative phenotype is characterized by a decrease in SMC markers and increased proliferation and migration, and is known to be involved in the development and progression of vascular diseases such as atherosclerosis [15]. Foam cell formation is a characteristic of early stage atherosclerosis, in which monocytes enter the arterial intima through the endothelium damaged by pro-inflammatory stimuli and differentiate into macrophages. Media VSMCs that undergo phenotypic switching also migrate to the intima and contribute to foam cell formation. It is known that SMC and macrophage absorb aggLDL, oxLDL and enzymatically modified LDL through various scavenger receptors to form foam cells [16]. They regulate foam cell formation in macrophages by several mechanisms including lipoprotein uptake, lipid efflux, inflammatory signaling, and endocytosis. In addition, it is known that lipoprotein uptake by Cholesterol and oxLDL in VSMC foam cell formation leads to activation of the signaling system by scavenger receptors and receptors related to inflammatory responses, which contributes to pathophysiology of atherosclerosis [17]. Lipid uptake in VSMCs transformation by cholesterol and oxLDL is known to involve scavenger receptors and receptors related to inflammatory responses. Among them, TLR4 has been shown to be involved in micropinocytosis and lipoprotein uptake during mmLDL treatment and mediates lipid accumulation in macrophages [18]. Also, minimally modified LDL (mmLDL) stimulation in macrophage induces phosphorylation of TLR4 and Syk, resulting in the activation of signaling pathways involved in lipoprotein uptake foam cell formation, and is known to contribute to atherosclerosis [19].

Although the understanding of SMC plasticity has been advancing in recent years, the mechanism has not been fully elucidated. To date, several key transcription factors and regulatory elements have been identified that play an important role in regulating SMC status. To understand the molecular mechanisms underlying SMC plasticity, several studies have focused on the role of epigenetics. A summary of transcription factors and HDAC binding through histone modification and DNA methylation indicates that phenotype modulation is reduced while histones are acetylated. DNA methylation (5mC) is associated with gene inactivation [20] [21]. It has been demonstrated that the HGPS mutant protein, progerin, alters histone methylation sites known to regulate heterochromatin, suggesting that under normal conditions, LaminA is involved in heterochromatin regulation [14]. Furthermore, it has been reported that the expression of DNA methyltransferases in atherosclerotic lesion [22]. After confirming that Zmpstes24 regulates the expression of TLR4 through DNA methylation, we searched for regulatory factors affecting DNA methylation. DNMT3a and DNMT3b, one of the subunits of DNA methyltransferase, it has been reported that be downregulated by oxLDL in VSMCs [23] [24]. As a result of investigating what factors regulate DNMT3a, as reported in scien in 2017, it was confirmed that MTA1 (Metastasis-associated protein 1) is a repression regulator of DNMT3a transcription [25]. In the present study, we determined the impact of the vascular aging-related Zmpste24 gene on vascular smooth muscle cell phenotypic switching and acceleration of atherosclerosis, and elucidated the underlying mechanisms.

II. MATERIALS AND METHODS

1. Cell Culture and treatments

Rat vascular smooth muscle cells (Rat VSMCs) were isolated from rat aorta. VSMCs were maintained at 37℃ in a humidified atmosphere containing 5% CO2 and 95% air. The VSMCs were cultured in Dulbecco's modified eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 5% penicillin (Gibco, Waltham, MA, USA). To induce atherosclerosis in VSMCs, the cells were cultured to 80% density, the culture medium was replaced with serum free medium for 1 h. After, the VSMCs were treated with 20 μg/ml water-soluble cholesterol (Sigma-Aldrich Corp., USA) for 48 h. To investigate the effect of Zmpste24 on high cholesterol conditions, VSMCs were transfected with control siRNA (100 μM) or Zmpste24 (100 μM), DNMT3a (100 μM) (Sigma, -Aldrich Corp., USA) and pCMV6-AC-GFP-hZmpste24-T7 constructs using Lipofectamine RNAiMAX (Thermo Fisher) following the manufacturer's instructions. 6 hours after transfection, VSMCs were replaced with culture medium for 24 hours. After replacing the culture medium with a serum-free medium for 1 hours, the VSMCs were treated with soluble cholesterol for 48 hours. VSMCs were treated with clathrin (Dynasore, 80 μM)-, caveolae (Filipin, 2.5 μM)-inhibitor prior to cholesterol treatment for 48 hours. VSMCs were incubated with TAK-242 (2, 3 μg/ml), a TLR4 specific inhibitor, or 5-az (10 μM), a DNA methyltransferase inhibitor, for 2 hours. VSMCs at passages 3-5 were used

for experiments.

2. Oil red O Staining

Cellular and tissue cholesterol deposits were visualized using oil red O staining. Oil Red O, diluted to 0.5% in Propylene glycol, was prepared. VSMCs were transfected with Zmpste24 siRNA prior to cholesterol treatment for 48 hours, afterwards the cells were washed two times with PBS, fixed for 10min in 10% formaldehyde, rinsed 3 times with distilled water, stained with 0.5 % oil red O solution for 2 hours at room temperature. Cells were washed with 85 % propylene glycol for 20 min and water for 3 times. Hematoxylin was used to stain cell for 5 sec. Image of the cells were photographed with microscope of Olympus BX53F (Olympus, Tokyo, Japan).

3. DNA extraction and Bisulfite modification and MSP assay

DNA was isolated from cells using a Quick-DNA^{TM} Miniprep Kit (ZYMO RESEARCH, Germany & EU) after VSMCs were treated according to the manufacturer's instructions. Purified genomic DNA (1 μg) was PCR amplified with the house-keeping gene GAPDH (F: 5'-GGGTGTGAACCACGAGAAATA-3', R: 5'-GGGTCTGGGATGGAAATTGT-3') as a control, and the PCR products were examined by electrophoresis. All purified genomic DNA (2 μg) from cells or tissues were bisulfite modified with the EZ DNA methylation Kit

(Streamlined bisulfite conversion of DNA, ZYMO RESEARCH, Germany & EU) according to manufacturer's instructions. The modified DNA 2 μl was PCR amplified with specific-unmethylated TLR4, specific-methylated TLR4 sequencing primers.

4. Chromatin immunoprecipitation polymerase chain reaction assay (ChIP-PCR assay)

ChIP-PCR assay was performed according to Hus et al. with modification [26]. Briefly, 1 % formaldehyde-treated nuclear lysates were subjected to chromatin immunoprecipitation with 5mC antibodies. The cross-linked chromatin complex was reversed in the presence of proteinase K and DNA fragments were purified. The DNA fragments of TLR4 was amplified by PCR using a pair of primers (F: 5'-GAGGACTGGGTGAGAAACGA-3', R: 5'-GAAACTGCCATGTCTGAGCA-3'). PCR condition was as follows: at 95 ℃ for 30 sec; at 52 ℃ for 30 sec; and at 72 ℃ for 30 sec. After 30 cycles of PCR, products were analyzed by 1.5 % agarose gel electrophoresis. For input (5 %) DNA, 25 μl aliquots of 500 μl samples were taken before immunoprecipitation.

5. Total RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Cells were harvested and total RNA was extracted by using a Ribospin kit (GeneAll, Seoul, Republic of Korea) according to the manufacturer's instruction. Total RNA from mice VSMCs was isolated using the QIAzol Lysis Reagent (Qiagen, Hilden, Germany). The total concentration of RNA was measured by using Nanodrop-2000/2000c (Thermo Scientific, Long Beach, NY, USA). 1 μg of total RNA samples were converted into complementary DNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). For RT-PCR, the cDNA was amplified using AccuPower® PCR PreMix (Bioneer, Daejeon, Republic of Korea) with primers. PCR products were separated by electrophoresis in a 1 ~ 3 % agarose gel. The primer sequences are shows in Table 1.

Table 1. Primer sequences for PCR

Gene	Primer sequence (5' - 3')
a-SM actin	F: CATCACCATCGGGAATCAACGC
	R: CTTAGAAGCATTTGCGGTGGAC
SM22 alpha	F: TGTTCCAGACTGTTGACCTC
	R: GTGATACCTCAAAGCTGTCC
Mac2	F: CCCGCTTCAATGAGAACAAC
	R: ACCGCAACCTTGAAGTGGTC
CD68	F: TCCTTCACGGAGACACCT
	R: GGCTGGGAACCATTAGTC
LDLR	F: AGGAGTGCAAGACCAACGAG
	R: TATCTTCACACTGGTGGCCG
SR-A	F: CCTTGATTTCATCAGTCCAGGAAC
	R: GTTGCTTTGCTGTAGATTCGCGG
CD36	F: GTGCAAAGAAGGAAAGCC
	R: CATCACTACTCCAACACC
TLR4	F: GAGGACTGGGTGAGAAACGA
	R: GAAACTGCCATGTCTGAGCA
Zmpste24	F: GGAAGTTGGGACACACAGTAA
	R: GAAGCGGAGGGTGTGAATAG
DNMT1	F: CAGAGGAGAGAGACCAGGATAA
	R: CTCCTGCATCAGCCCAAATA
DNMT3a	F: CCACCAGGTCAAACTCCATAAA
	R: TGTTCCTGTTTGTGCTCCTATC
DNMT3b	F: GATCCTGACCTGGAAGAGTTTG
	R: GGAGTTCGACTTGGTGGTTATT
MAT1	F: GGAGCGTGAGGATTTCTTCTT
	R: ACGTTTCTGCTGGACGTATC

6. Western Blot analysis

The cells were washed twice in cold PBS and protein was extracted from the VSMCs and lysed with RIPA buffer (Biosesang, Korea) containing 150 mM NaCl, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, 500 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8.0) and a protease and phosphatase inhibitor cocktail (Thermo scientific Inc., USA) on ice for 30 min. Subsequently, cells with lysis buffer were centrifuged at 14,000 RPM for 20 min at 4 ℃ and supernatants were transferred to new tubes. Protein concentrations were measured by bicinchoninic acid (BCA, Sigma, USA) protein assay and 25 μg samples of the cell lysates were electrophoresed on SDS-PAGE gels and proteins were transferred onto polyvinylidene difluoride membranes (ATTO, Japan) and blocked with 5 % non-fat milk at room temperature for 1 h. And membranes were washed three times with TBS-T for 5 min. The membranes were incubated with primary antibodies for overnight at 4 ℃. The membranes were washed 3 times with TBS-T every 10 min and then membranes were incubated with HRP-conjugated secondary antibodies (Gendepot) for 1 hour at room temperature. Signals were detected using enhanced chemiluminescent reagent (GE Healthcare, UK) and were quantified using Image J software (National Institutes of Health, USA). All blots were probed using GAPDH as a loading control on the same membrane and analyzed with image J software (National Institutes of Health, Bethesda, MD, USA)

7. Animal studies

All animal care and experiments were approved by the local Institutional Animal Care and Use Ethics Committee (IACUC: 2018-0073) and Yonsei University Health System in ABMRC. Mice were tested using Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice, which were produced in collaboration with Professor Hanwoong Lee's laboratory at Yonsei University. The identification of ApoE$^{-/-}$ mice and Zmpste24 $^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ were verified by PCR analysis using DNA extracted from mice tail samples. Cut tails were melted using mixture of Proteinase K from tail lysis buffer (50 mM Tris (pH 8.0), 50 mM EDTA, 0.5 % SDS), melted at 56 ℃ using heat block with 600 RPM shaking for 4 ~ 8 hours. The gDNA was used in PCR; PCR was performed using the AccuPower PCR premix (Bioneer, Daejeon, Korea) and cycling conditions of genotyping were SM22aCre^{+}: 95 ℃ for 4 min, 29 cycles of 95 ℃ for 30 sec, 60 ℃ for 30 sec, 72 ℃ for 30 sec and 72 ℃ for 5 min for final extension, ApoE$^{-/-}$: 94 ℃ for 5 min, 29 cycles of 94 ℃ for 30 sec, 60 ℃ for 1 min, 72 ℃ for 30 sec and 72 ℃ for 5 min for final extension, Zmpste24$^{F/F}$: 95 ℃ for 5 min, 35 cycles of 95 ℃ for 30 sec, 65 ℃ for 30 sec, 72 ℃ for 30 sec and 72 ℃ for 5 min for final extension. The primers for the model were SM22aCre^{+} (RF67: 5'-CTCAGAGTGGAAGGCCTGGTT-3', RF90: 5'-CACACCATTCTTCAGCCACA-3', SC135: 5'-GGCGATCCCTGAACATGTCC-3'), ApoE$^{-/-}$ (F: 5'-AGAACTGACGTGAGTGTCCA-3', R: 5'-GTTCCCAGAAGTTGAGAAGC-3', Neo: 5'-GCTTCCTCGTGCTTTACGGTA-3'), Zmpste24$^{F/F}$ (sgZmpR2-1R: 5'-

CTGAGGACTGCGCTGAACAC-3', ZmpR1-F2: 5'-AAGCCCTGGGATGACCTCA-3'). The PCR products were analyzed by 1.5 ~ 3 % agarose gel electrophoresis. 8-week-old $ApoE^{-/-}$ mice and $Zmpste24^{F/F}$ $SM22\alpha Cre^{+}$ $ApoE^{-/-}$ mice were intraperitoneal injection of total 25 μg Tamoxifen [27] for 5 days and fed a high cholesterol diet (16 % gm fat, 12.5 % gm cholesterol, D12336, Research Diets, New Brunswick, USA) to induce atherosclerosis for 8 weeks. After waiting for 8 weeks' diet period, the animals were sacrificed for obtained the aorta, heart and blood samples. And for RNA and protein was isolated in aorta.

8. Immunofluorescence microscopy

The aortic arch sections were fixed in 10 % formalin and processed in 4 μm-thick sections for immunofluorescence staining. Paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded ethanol and distilled water. For antigen retrieval, samples were heated in citrate buffer (pH 6.0, Dako, Japan) for 40 min. After cooling at 4 °C for 10 min, the sections were washed with PBS-T, treated with 3% H2O2 in PBS, 3 times washed with PBS-T, and incubated 0.075 % Glycine in PBS for 10 min at room temperature. The sections were washed 3 times for 5 min with PBS-T, and were permeabilized for 10 min on 0.4 % TritonX-100 in PBS-T, then blocked for 30 min in PBS-T containing 1 % bovine serum albumin (BSA) at room temperature. Samples were then incubated in the following primary antibodies overnight at 4 °C: anti-Lamin A (Santa Cruz). The sections were washed 3 times for 5 min with PBS-T and incubated for 1 h at room temperature in the dark using

Alexa FluorTM594-goat anti-mouse IgG antibody. The sections were rewashed 3 times by every 5 min with PBS-T and were mounted using DAPI-containing mounting medium (VECTASHIELD, Burlingame, USA). The immunoreactivity signals were visualized by confocal laser scanning microscope LSM780(Carl Zeiss, Germany).

9. Statistical analysis

Data were expressed at the mean ± SEM. The significance of differences was estimated by one-way ANOVA followed by Bonferroni's post-hoc comparisons test. $P<0.05$ was considered statistically significant. All data analysis was performed with commercially available GraphPad Prism 5 software. Image J (version 10.0, NIH, Bethesda, MD, USA) and GraphPad Prism (version 7.04, Prism Software, San Diego, CA, USA) were used for data analysis and visualization. All data are expressed as mean ± SEM from at least four independent experiments. Statistical comparisons were performed using Student's t-test or one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Statistical significance was set at $P < 0.05$.

III. RESULTS

Part I. Effects of Zmpste24 knockdown on cholesterol-induced VSMC phenotypic switching

1. Effect of Cholesterol on VSMC phenotypic switching

First, the effect of cholesterol on phenotypic switching in VSMCs were investigated. The VSMC were treated with 20 μg/ml of cholesterol time-dependently (0, 1, 3, 6, 12, 24, 48 h) in serum free DMEM. Previous studies, the above dose was used because the 20 μg/ml of cholesterol showed the significantly VSMCs phenotypic switching. The expression of the phenotypic switching marker was checked at the RNA level. The expression of a-SM actin mRNA, known as an SMC marker, was significantly decreased from 6 hours, but an increase in the expression of CD68 mRNA, known as a macrophage marker at 48 hours (Figure 1). Therefore, 48 hours was regarded as the time when the most meaningful change in SMC and macrophage markers occur and thus, the cholesterol treatment time was set as 48 hours. These data suggest that cholesterol significantly induced VSMCs trans-differentiation of rat VSMCs.

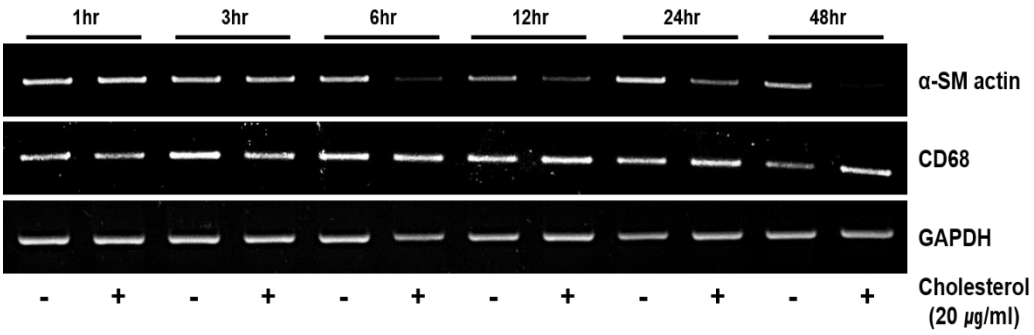
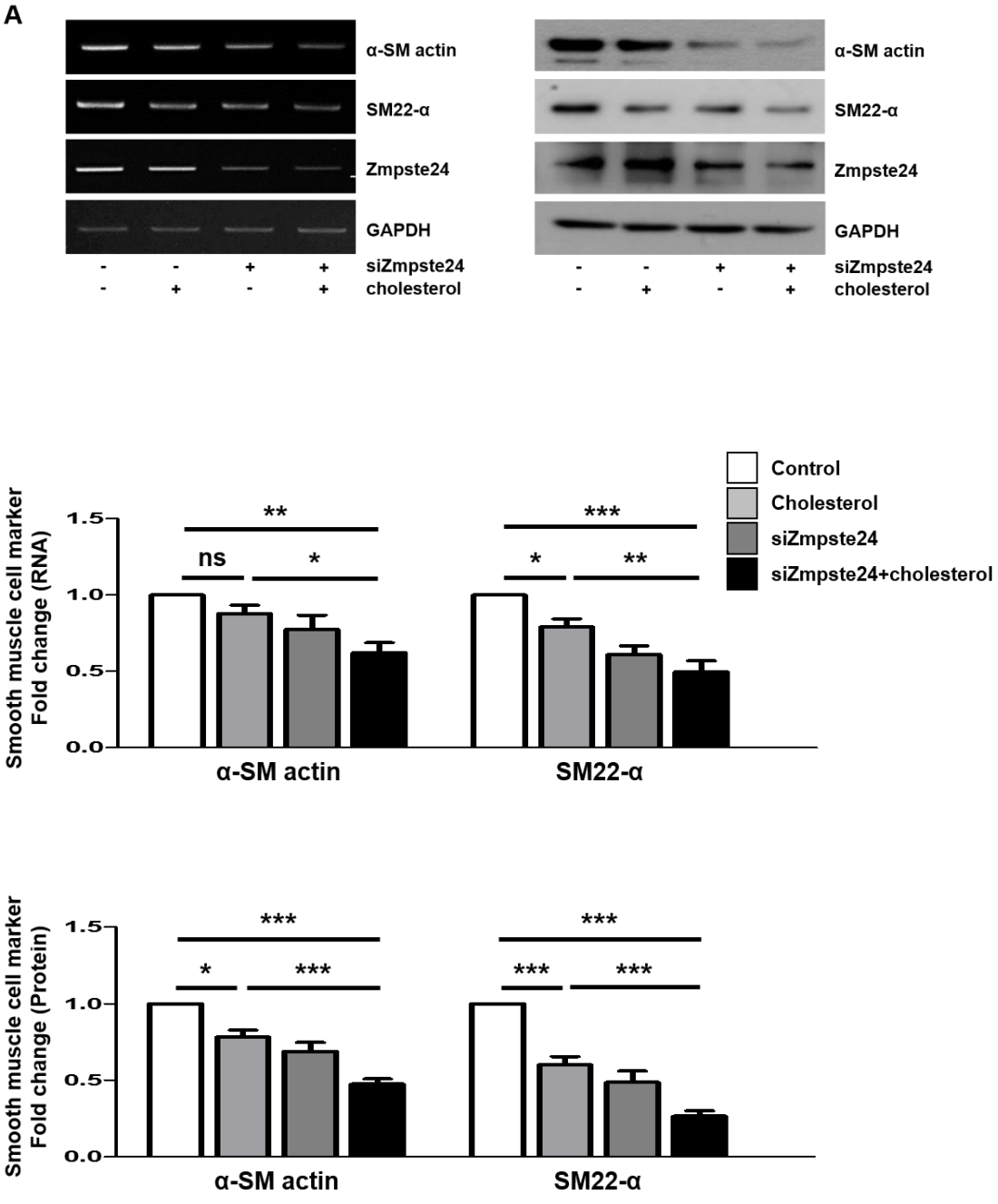


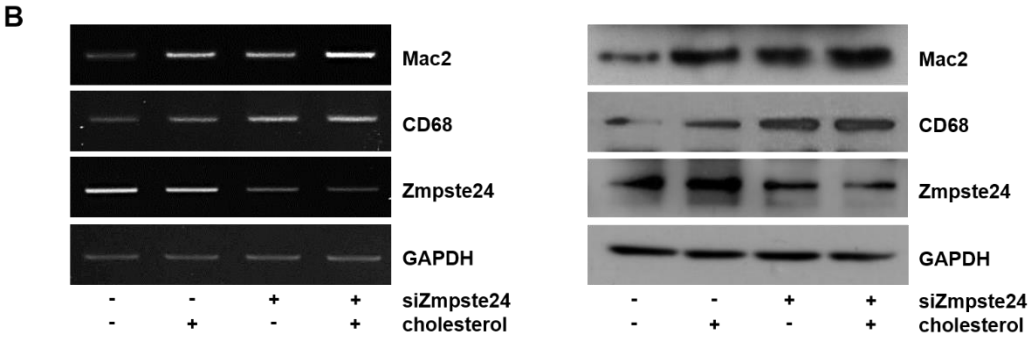
Figure 1. Cholesterol induces VSMCs phenotypic switching.

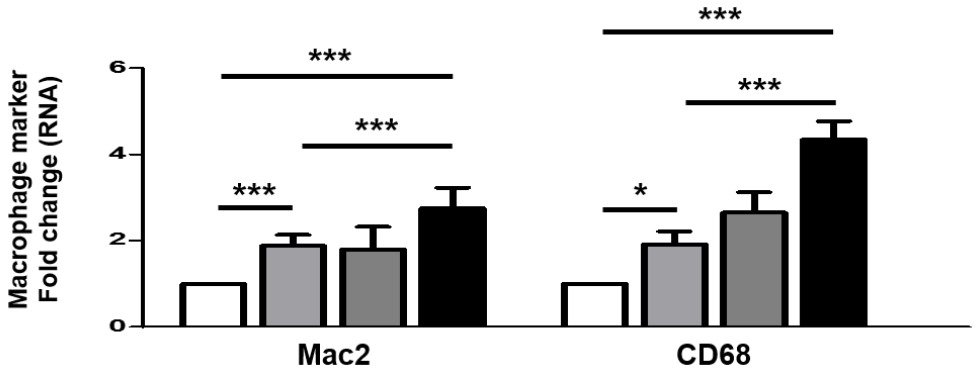
The vascular smooth muscle cells were treated with cholesterol (20 μg/ml) time-dependently (0, 1, 3, 6, 12, 24, 48 h). The SMC marker a-SM actin and the macrophage marker CD68 mRNA expression was measured by RT-PCR. The quantification was done by normalization of the density to that of GAPDH.

2. Deficiency of Zmpste24 accelerates VSMC phenotypic switching and VSMC foam cell formation

Next, to investigated whether Zmpste24 plays an important role in cholesterol-induced VSMC phenotypic switching and VSMC foam cell formation, the VSMC was treated with Zmpste24 siRNA (100 μM) prior to cholesterol (20 μg/ml) treatment for 48 hours. The VSMC treated with Zmpste24 siRNA (100 μM) prior to cholesterol treatment effectively reduced the mRNA and protein expression level of a-SM actin and SM22a, known as an SMC marker, compared to only cholesterol treatment group (Figure 2A). On the other hands, mRNA and protein expression level of Mac2 and CD68, known as a macrophage marker, significantly increased. These results, suggest deficiency of Zmpste24 accelerates cholesterol-induced VSMC phenotypic switching (Figure 2B). Next examined the effect of Zmpste24 on cholesterol-induced foam cell formation in VSMC. The VSMCs were treated with Zmpste24 siRNA (100 μM) prior to cholesterol (20 μg/ml) treatment for 48 hours. Visualization of cholesterol deposition on VSMCs was performed by oil red O staining. As a result, it was confirmed through staining that foam cell formation was increased when Zmpste24 siRNA and cholesterol were co-treated compared to cholesterol alone treatment (Figure 3). Therefore, these results suggest that the deficiency of Zmpste24 in cholesterol-treated VSMCs accelerates phenotypic switching and foam cell formation.







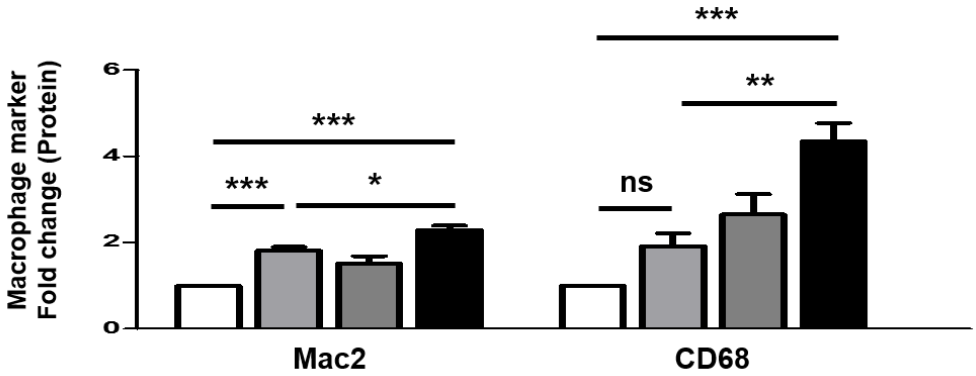


Figure 2. Zmpste24 regulates cholesterol-induced VSMC phenotypic switching.

Vascular smooth muscle cells treated with Zmpste24 siRNA prior to cholesterol treatment for 48 hours.

(A) The expression of a-SM actin and SM22a were evaluated by RT-PCR and western blot. The quantification was done by normalization of the density to that of GAPDH. (B) The expression of Mac2 and CD68 were evaluated by RT-PCR and western blot. The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant.

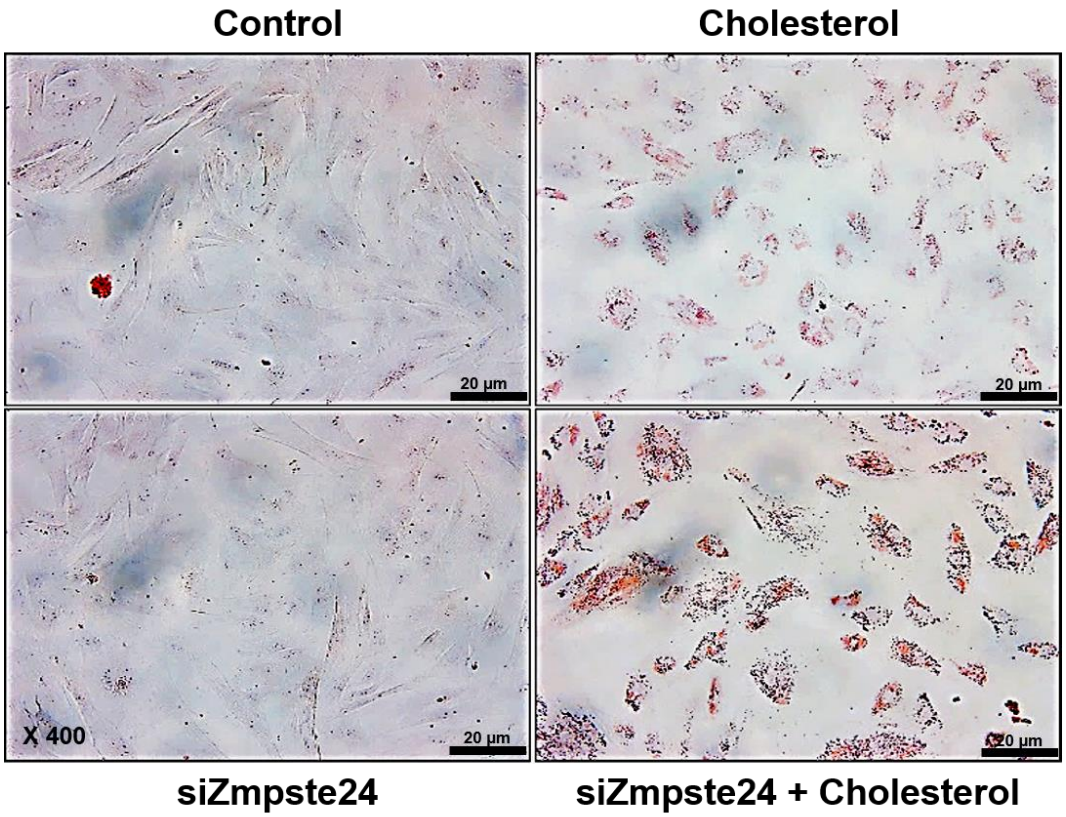


Figure 3. Zmpste24 regulates cholesterol-induced VSMC foam cell formation.

The vascular smooth muscle cells were treated with Zmspte24 siRNA, and then subjected to cholesterol treatment for 48 hours. Representative image show the cholesterol accumulation on VSMCs was assessed by oil red o staining. Cholesterol deposition was increased in VSMCs treated with Zmpste24 siRNA and cholesterol compared to those treated with cholesterol alone. Image of the cells were photographed using Olympus BX53F microscope.

Part II. Effects of Zmpste24 on cholesterol uptake by TLR4

3. Clathrin-, caveolae-mediated endocytosis is not involved in cholesterol uptake in VSMCs

It has been reported that various lipid uptake mechanisms have been reported to play an important role in foam cell formation, we first examined endocytosis related to lipid uptake in cholesterol-treated VSMCs. VSMCs were treated with clathrin (Dynasore, 80 μM)-, caveolae (Filipin, 2.5 μM)-inhibitor prior to cholesterol treatment for 48 hours, using oil red O staining confirm the effect on foam cell formation. As a results, it was confirmed that both clathrin-, caveolae-inhibitor involved in endocytosis did not inhibit cholesterol uptake (Figure 4). These results indicate that cholesterol uptake through endocytosis did not affect foam cell formation under our experimental conditions.

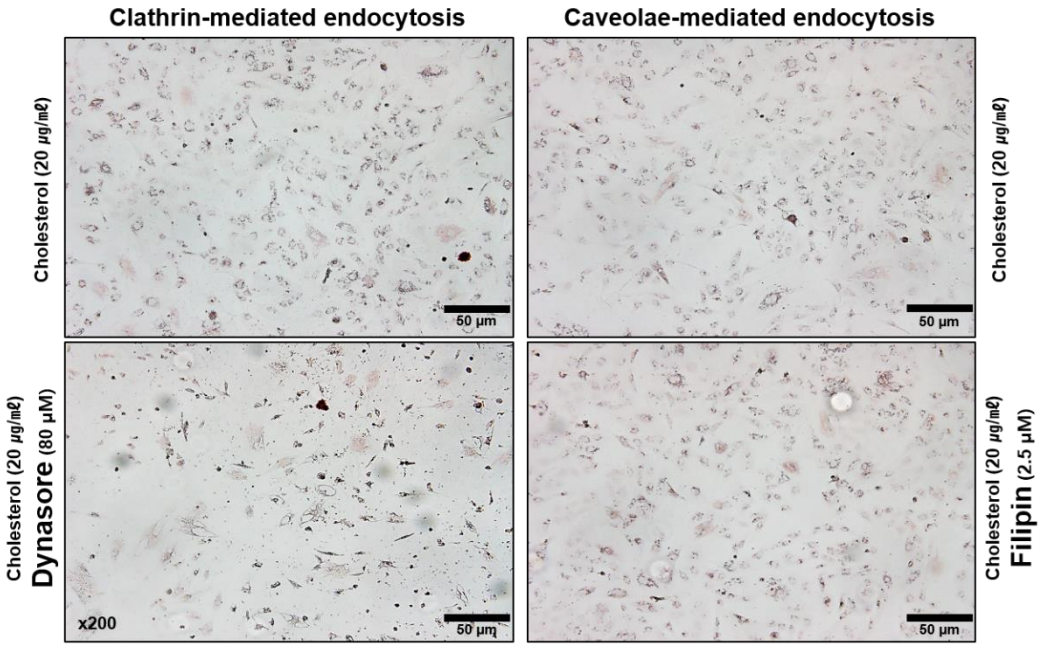
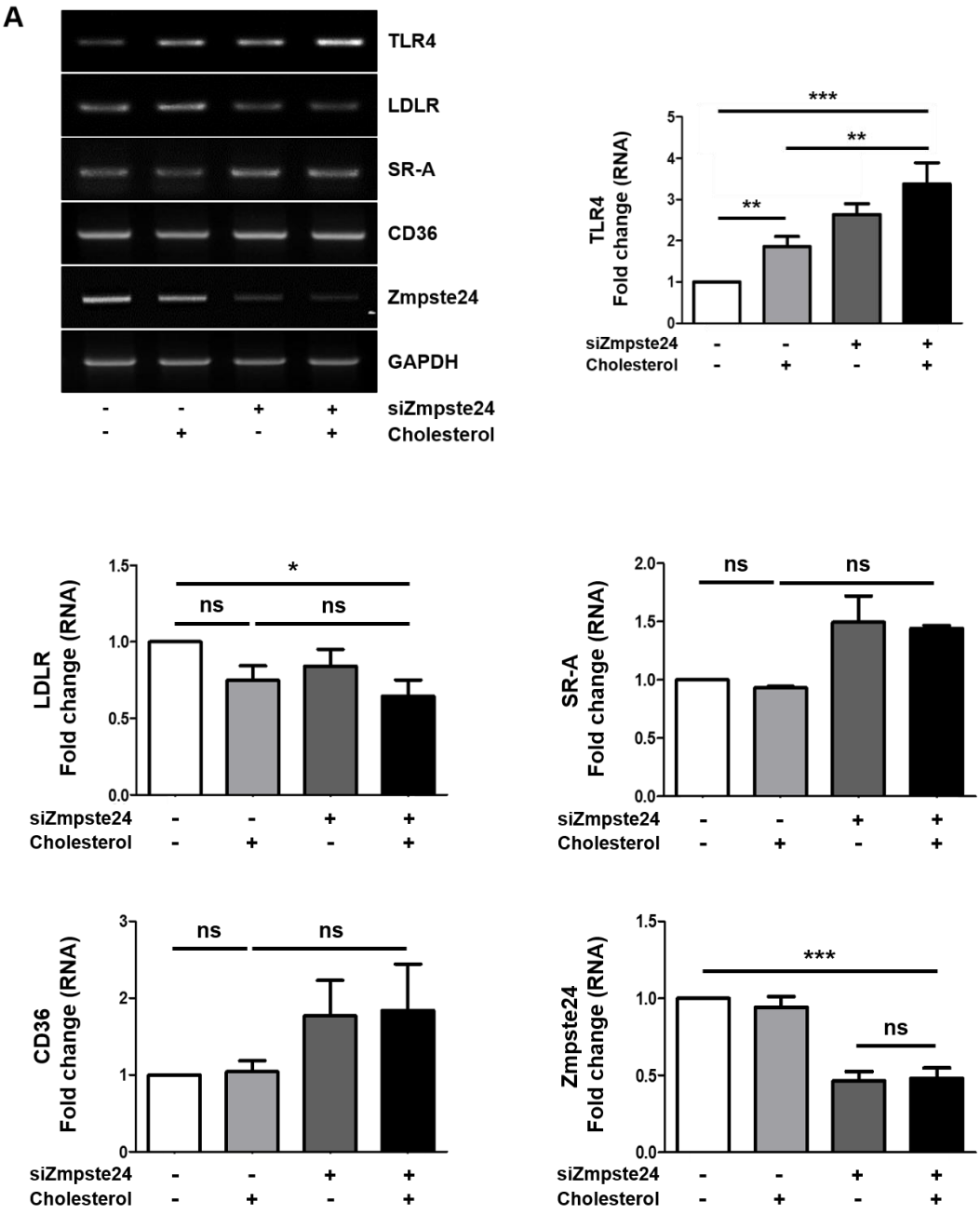


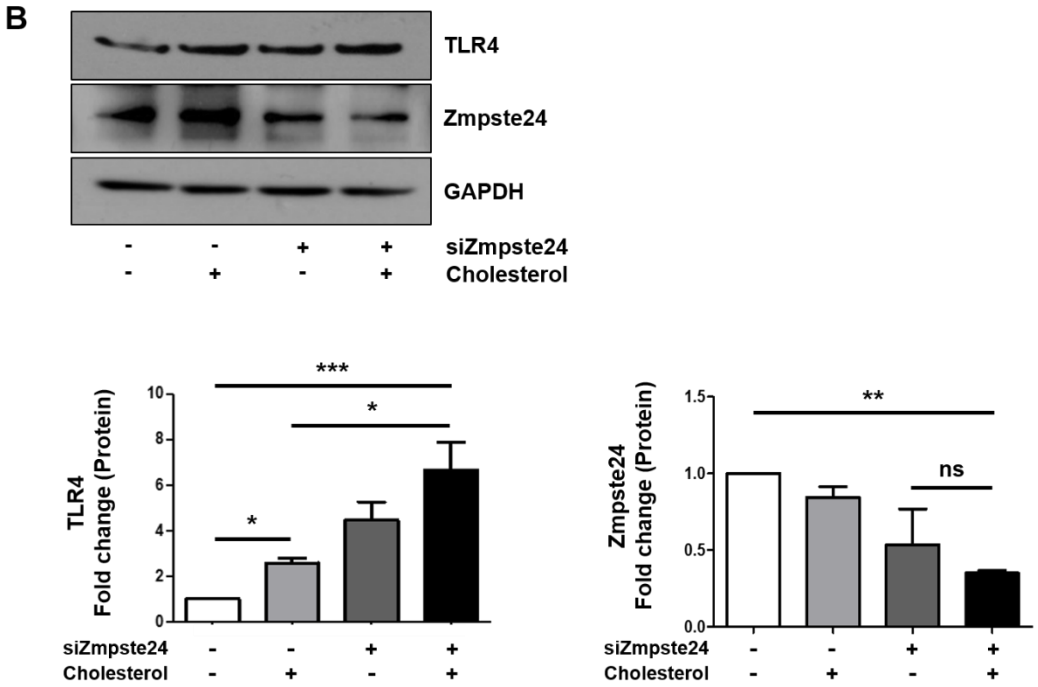
Figure 4. Inhibition of Clathrin-, Caveolae-mediated endocytosis does not inhibit cholesterol accumulation in VSMCs.

The vascular smooth muscle cells were pre-treated with Clathrin (Dynasore, 80 μM)-, Caveolae (Filipin, 2.5 μM)-mediated endocytosis inhibitor, and then treated with cholesterol for 48 hours. Representative images after oil red O staining showed that cholesterol accumulation was not mediated through Clathrin-, Caveolae-mediated endocytosis. Image of the cells were photographed with Olympus BX53F microscope.

4. Zmpste24 regulates TLR4 expression in cholesterol-treated VSMCs

In the next experiment, I was investigated the types of scavenger receptors and inflammatory signal receptors that mediate the uptake of cholesterol in VSMC treated with Zmpste24 siRNA. The vascular smooth muscle cells were pre-treated with Zmspte24 siRNA or pCMV6-AC-GFP-hZmpste24-T7 vector, and then cholesterol treatment was done for 48 hours, after which RT-PCR was performed. As a result, in the case of LDLR, SR-A, and CD36, receptors related to lipoprotein uptake, there was no significant change in both the cholesterol-only treatment group and the Zmpste24 siRNA and cholesterol co-treated group. On the other hand, TLR4 was significantly increased in the cholesterol-only treatment group compared to control and there was a significant, further increase in TLR4 expression in the Zmpste24 siRNA and cholesterol co-treated compared to cholesterol-only treatment group (Figure 5A). Also, Zmpste24 deficiency increased TLR4 expression at the protein level to the similar extent of the RT-PCR experiment (Figure 5B). In addition, to check whether Zmpste24 critically regulates TLR4 expression, I was overexpressed Zmpste24 using pCMV6-AC-GFP-hZmpste24-T7 vector (Figure 5C). As a result, it was found that overexpression of Zmpste24 reduced the expression of TLR4. In conclusion, this suggests that Zmpste24 regulates cholesterol uptake through TLR4 expression in cholesterol-treated VSMCs. These results show that Zmpste24 deficiency increases VSMC uptake of cholesterol through the increased expression of TLR4.





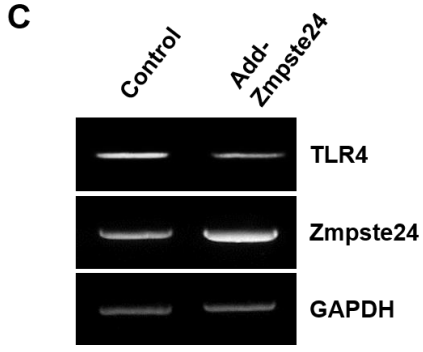


Figure 5. Zmpste24 regulates TLR4 expression in VSMCs.

Vascular smooth muscle cells were pre-treated with Zmpste24 siRNA or pCMV6-AC-GFP-hZmpste24-T7 vector, and then 20 μg/ml cholesterol treatment for 48 hours.

(A) The expression level of LDLR, SA-R, CD36, TLR4, Zmpste24 were assessed by RT-PCR. The quantification was done by normalization of the density to that of GAPDH. (B) The expression level of TLR4, Zmpste24 were assessed by western blot. The quantification was done by normalization of the density to that of GAPDH. (C) The expression level of TLR4 and Zmpste24 were assessed by RT-PCR. Values are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant.

5. Expression of TLR4 affects cholesterol uptake in VSMC

To determine whether TLR4 is directly involved in cholesterol uptake, it was verified using TAK-242, a TLR4 specific inhibitor. After TAK-242 treatment in a dose-dependent manner, cholesterol accumulation was confirmed by oil red O staining. As a result, it can be clearly seen that TLR4 affects cholesterol uptake in VSMC, as the degree of cholesterol accumulation decreases in a dose-dependent manner (Figure 6).

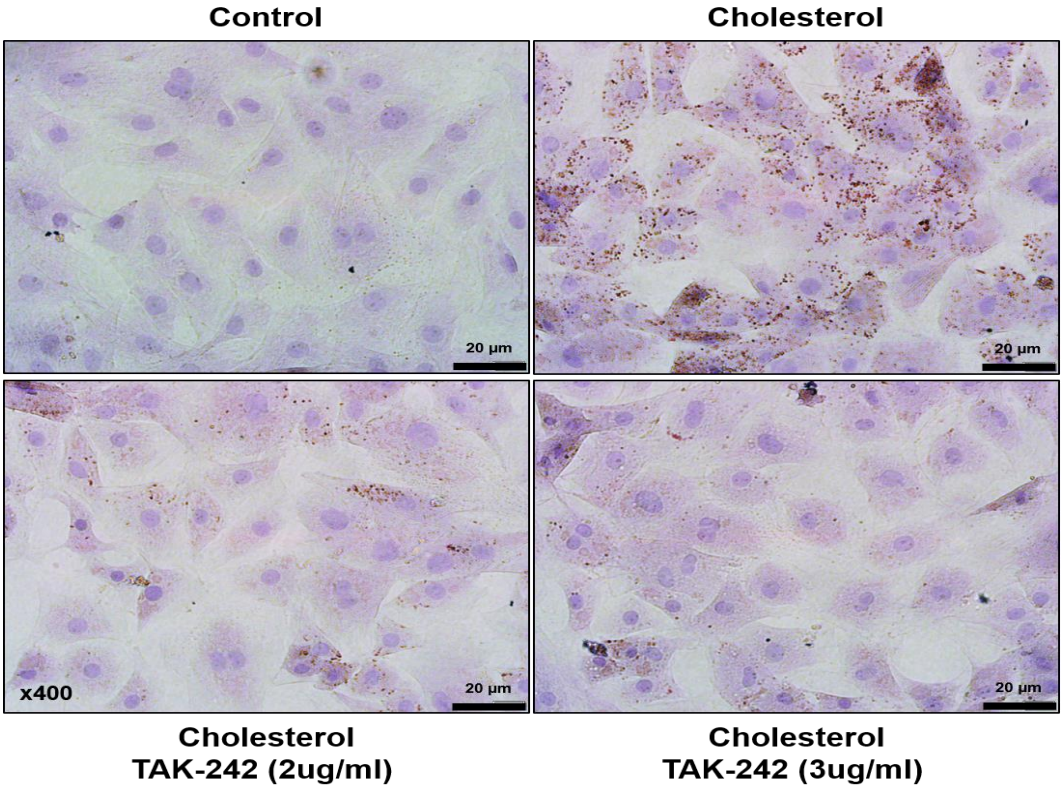


Figure 6. TAK-242, a TLR4-specific inhibitor inhibits cholesterol accumulation in VSMCs.

Vascular smooth muscle cells were pre-treated with various concentration (2, 3 μg/μl) of TLR4-specific inhibitor, TAK-242 for 2 hours, and subsequently treated with cholesterol for 48 hours. Representative image show the cholesterol accumulation on VSMCs was assessed by oil red o staining. Image of the cells were photographed using Olympus BX53F microscope.

6. Deficiency of Zmpste24 increases cholesterol uptake via TLR4 in VSMC

The next step of the experiment was to confirm whether or not Zmpste24 had an effect on cholesterol uptake by TLR4. Cholesterol accumulation was assessed by oil red o staining in cholesterol treated VSMC treated with Zmpste24 siRNA co-treated with TLR4-specific inhibitor, TAK-242. When TAK-242 was not treated, the number of foam cells increased more in the group treated with Zmpste24 siRNA and cholesterol than in the cholesterol-treated group. However, co-treatment with TAK-242 resulted in the cholesterol accumulation in the Zmpste24 siRNA and cholesterol co-treated groups to a similar degree to that in the cholesterol treatment group (Figure 7). These results suggest that an increased expression of TLR4 plays an important role in VSMC foam cell formation in Zmpste24 deficiency.

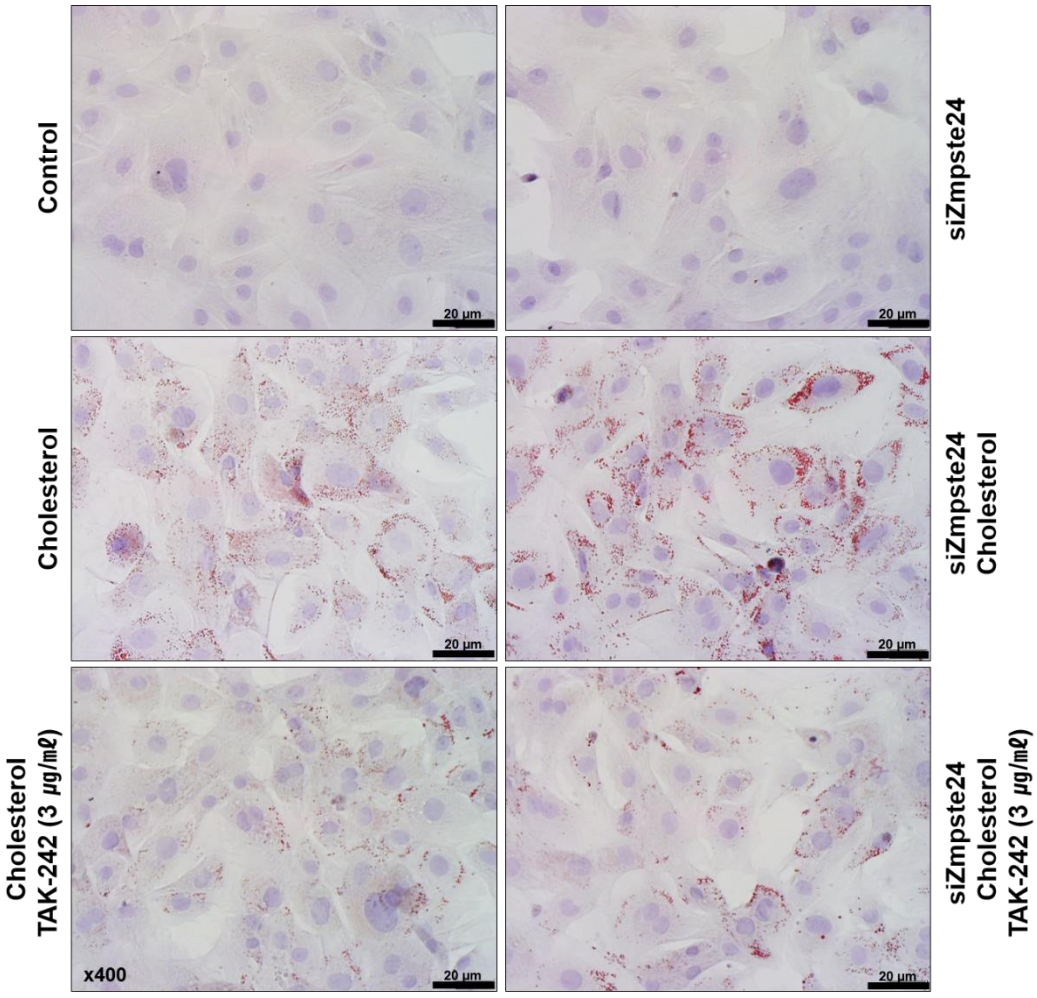


Figure 7. TLR4 accelerates cholesterol uptake in VSMCs.

Vascular smooth muscle cells were transfected with Zmpste24 siRNA, pre-treated with TLR4-specific inhibitor 3 μg/μl TAK-242 for 2 hours, and then treated with cholesterol for 48 hours. Representative image show the cholesterol accumulation on VSMCs was assessed by oil red o staining. Image of the cells were photographed with Olympus BX53F microscope.

Part III. Effects of Zmpste24 on modulating TLR4 expression through epigenetic modification

7. TLR4 genes are in methylated state in normal VSMCs

In order to understand the underlying molecular mechanisms of SMC, many studies have recently been conducted in relation to epigenetics [20] [21]. DNA methylation (5mC) is associated with gene inactivation. Therefore, I examined the degree of methylation of TLR4 in normal SMC with or without Zmpste24 and cholesterol by MSP analysis. As a result, it was confirmed that the amount of methylated-TLR4 was higher than that of unmethylated-TLR4 in VSMCs under normal conditions (Figure 8A and 8B). These shows that TLR4 expression is inhibited by methylation under normal conditions.

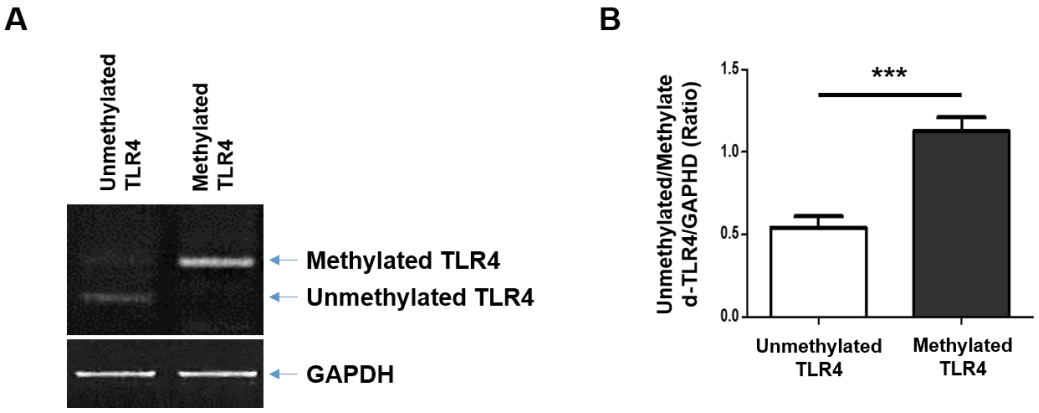


Figure 8. TLR4 genes are methylated in normal VSMCs.

DNA was isolated normal vascular smooth muscle cells. Isolated DNA was bisulfite modified. The modified DNA was PCR amplified with specific-unmethylated TLR4, specific-methylated TLR4 sequencing primers.

(A and B) The expression level of unmethylated TLR4, methylated TLR4 was analyzed by RT-PCR. The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. ***, $p < 0.001$

8. TLR4 expression regulates via epigenetic modification in normal VSMC

As TLR4 gene is in methylated condition in normal VSMC, I investigated whether demethylation by treatment with 5-az, a DNA methyltransferase inhibitor, regulates the expression of TLR4. As a result, the expression of TLR4 was increased in the group treated with 5-az compared to the control, indicating that TLR4 expression in normal VSMC is regulated through epigenetic modification (Figure 9).

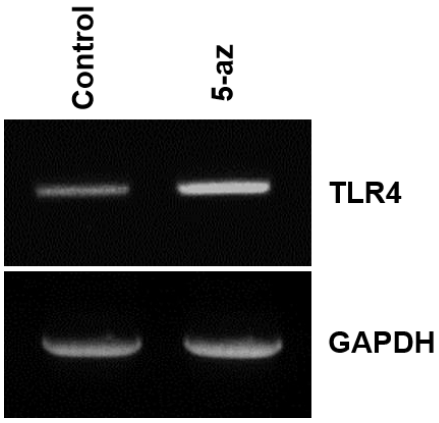


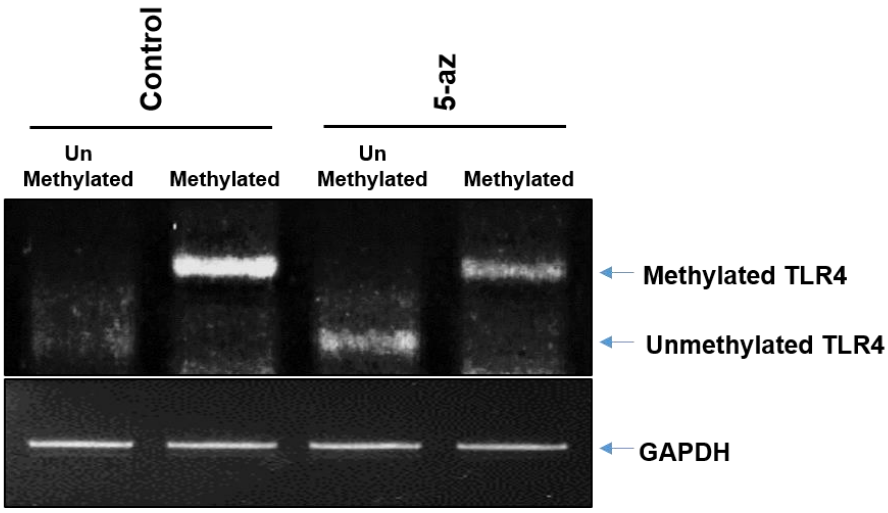
Figure 9. TLR4 expression upregulates by 5-az treatment in normal VSMCs.

Vascular smooth muscle cells were treated 5-aza for 48 hours and total RNA was isolated in cells. The expression level of TLR4 was assessed by RT-PCR.

9. Zmpste24 regulates DNA methylation in VSMC

To evaluate whether Zmpste24 critically regulates TLR4 expression through methylation, MSP assay was performed after VSMCs were pretreated 5-az and Zmpste24 siRNA. As a result, the control group had more methylated TLR4 with low expression of TLR4 expression, whereas in the 5-az (Figure 10A) and Zmpste24 siRNA-treated group (Figure 10B), methylated TLR4 decreased while um-methylated TLR4 increased, resulting in increased TLR4 expression. These data suggest that Zmpste24 deficiency regulates TLR4 expression by demethylation of the TLR4 gene.

A



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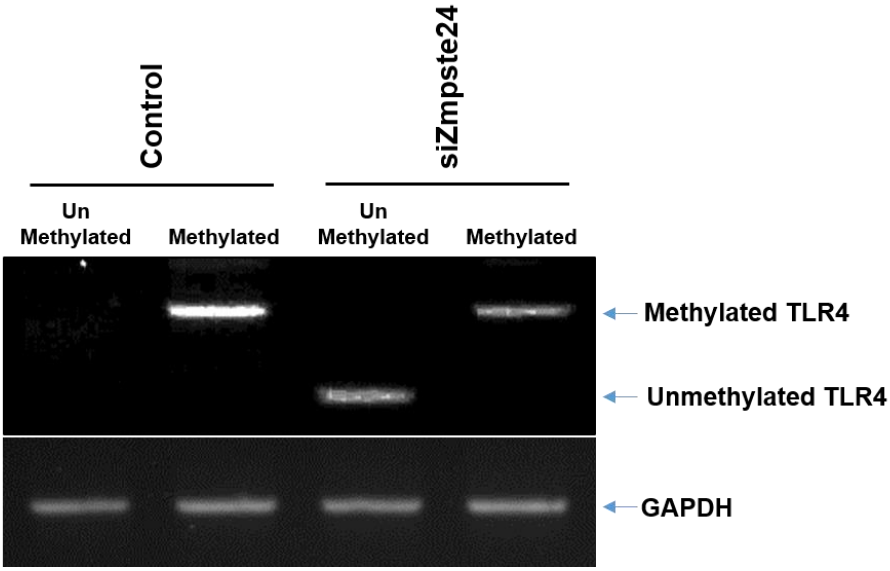


Figure 10. Zmpste24 deficiency decreases methylated TLR4 expression in normal VSMCs.

Vascular smooth muscle cells were treated with 5-az or Zmpste24 siRNA, and total DNA was isolated from the cells. The isolated DNA was bisulfite modified.

(A) Modified DNA from 5-az-treated samples was PCR amplified with specific unmethylated TLR4, specific methylated TLR4 sequencing primers. Expression levels of unmethylated TLR4 and methylated TLR4 were analyzed by RT-PCR. The quantification was done by normalization of the density to that of GAPDH. (B) The modified DNA of the Zmpste24 deficient sample was PCR amplified with specific unmethylated TLR4 and specific methylated TLR4 sequencing primers. Expression levels of unmethylated TLR4 and methylated TLR4 were analyzed by RT-PCR. The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant.

10. Zmpste24 regulates TLR4 expression through DNA methylation in cholesterol-treated VSMC

To confirm that the expression of TLR4 by Zmpste24 is regulated by DNA methylation, ChIP-PCR assay was performed. IP was performed using the target antibodies to 5-methylcytosine and then PCR was performed using the TLR4 primer. 5-Methylcytosine is a methylated molecule of cytosine©, which plays various biological roles in regulating gene transcription, etc. When IP is performed using 5mC antibody, only a DNA-Protein fragment with methylated cytosine is obtained. Subsequently, the precipitated DNA was purified and PCR was performed. As a result, the expression of TLR4 was significantly reduced even when Zmpste24 siRNA alone was treated compared to the control, and the expression of TLR4 was significantly reduced when co-treated with Zmpste24 siRNA and cholesterol than when treated with cholesterol alone (Figure 11A and 11B). These data strongly suggest that Zmpste24 regulates the expression of TLR4 through DNA methylation.

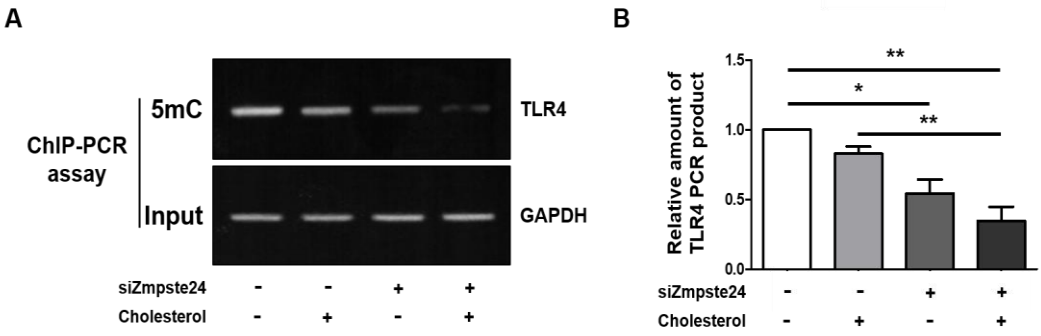


Figure 11. Deficiency of Zmpste24 decrease methylated TLR4 expression in cholesterol-treated VSMCs.

Vascular smooth muscle cells were treated with Zmpste24 siRNA prior to cholesterol treatment for 48 hours.

(A) Cross-linked cell lysates were subjected to ChIP-PCR analysis with anti-5mC antibody. RT-PCR was performed with ChIP primer as listed in Materials and Methods. The results present the means of five independent experiments. (B) The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$

11. Zmpste24 regulates DNMT3a expression in cholesterol-induced VSMC

Previously, it has been reported that DNMT1, DNMT3a, and DNMT3b, one of the subunits of methyltransferase related to DNA methylation, are downregulated by oxLDL in VSMC [15] [16]. Therefore, I investigated whether the expression of DNMT1, DNMT3a, and DNMT3b was regulated by cholesterol. As a result, the regulatory factor reduced by cholesterol was DNMT3a, and it was confirmed that the expression of DNMT3a was reduced further when cholesterol was co-treated with Zmpste24 siRNA (Figure 12A). In addition, it was confirmed that overexpression using pCMV6-AC-GFP-hZmpste24-T7 vector increased the expression of DNMT3a as opposed to knockdown (Figure 12B). From this, it was found that Zmpste24 regulates the expression of DNMT3a in cholesterol-induced VSMC.

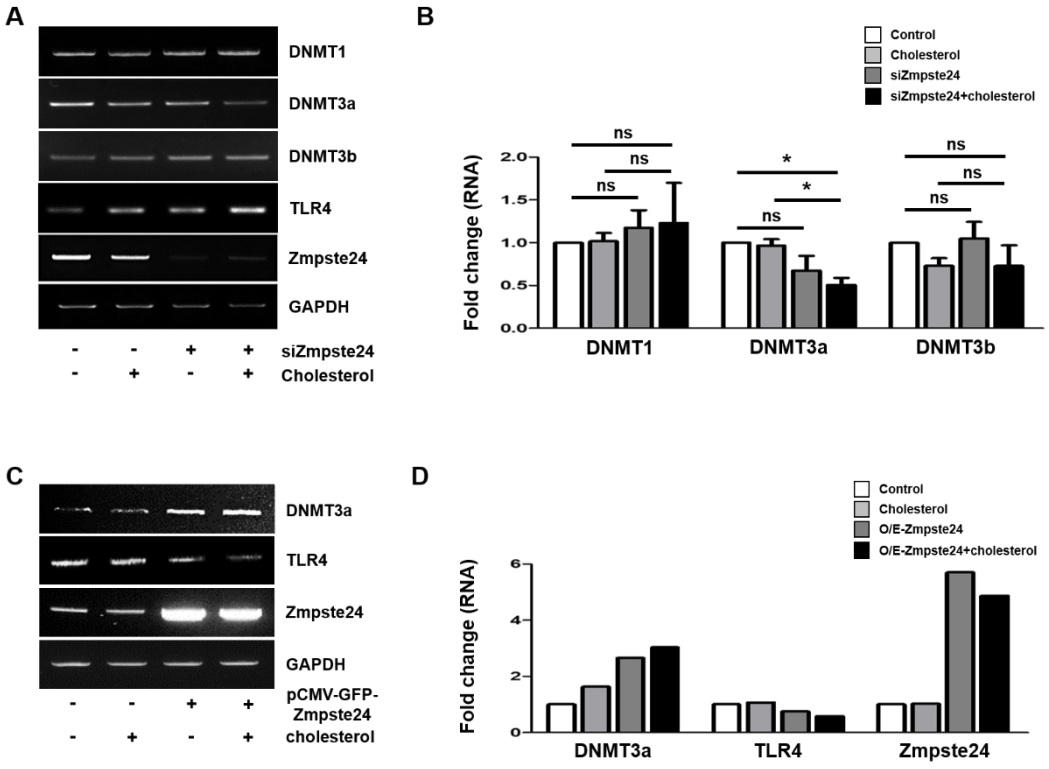


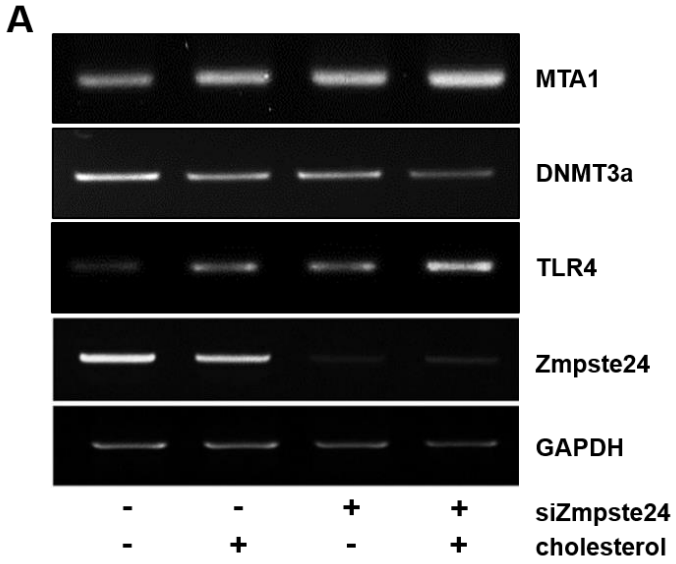
Figure 12. Zmpste24 regulates DNMT3a expression.

The vascular smooth muscle cells were pre-treated with Zmpste24 siRNA or pCMV6-AC-GFP-hZmpste24-T7 vector, and then cholesterol treatment for 48 hours.

(A and B) The expression level of DNMT1, DNMT3a, DNMT3b, TLR4, Zmpste24 were assessed by RT-PCR. The quantification was done by normalization of the density to that of GAPDH. (C and D) The expression level of DNMT3a, TLR4, Zmpste24 were assessed by RT-PCR. Values are mean ± SEM. *, $p < 0.05$; ns, not significant.

12. Zmpste24 regulates MTA1 expression in cholesterol-treated VSMC

As a result of checking what factors regulate DNMT3a, it was confirmed that MTA1 (Metastasis-associated protein 1) is a repression regulator of DNMT3a transcription [25]. Therefore, I decided to first check the expression levels of MTA1 and DNMT3a by transfecting Zmpste24 siRNA into VSMC to see if MTA1 is regulated by Zmpste24. As a result, it was confirmed that MTA1 increased when Zmpste24 deficiency the expression increased more significantly when co-treated with cholesterol (Figure 14A and 14B). However, in the present experiment, the causality between MTA1 and DNMT3a could not be confirmed.



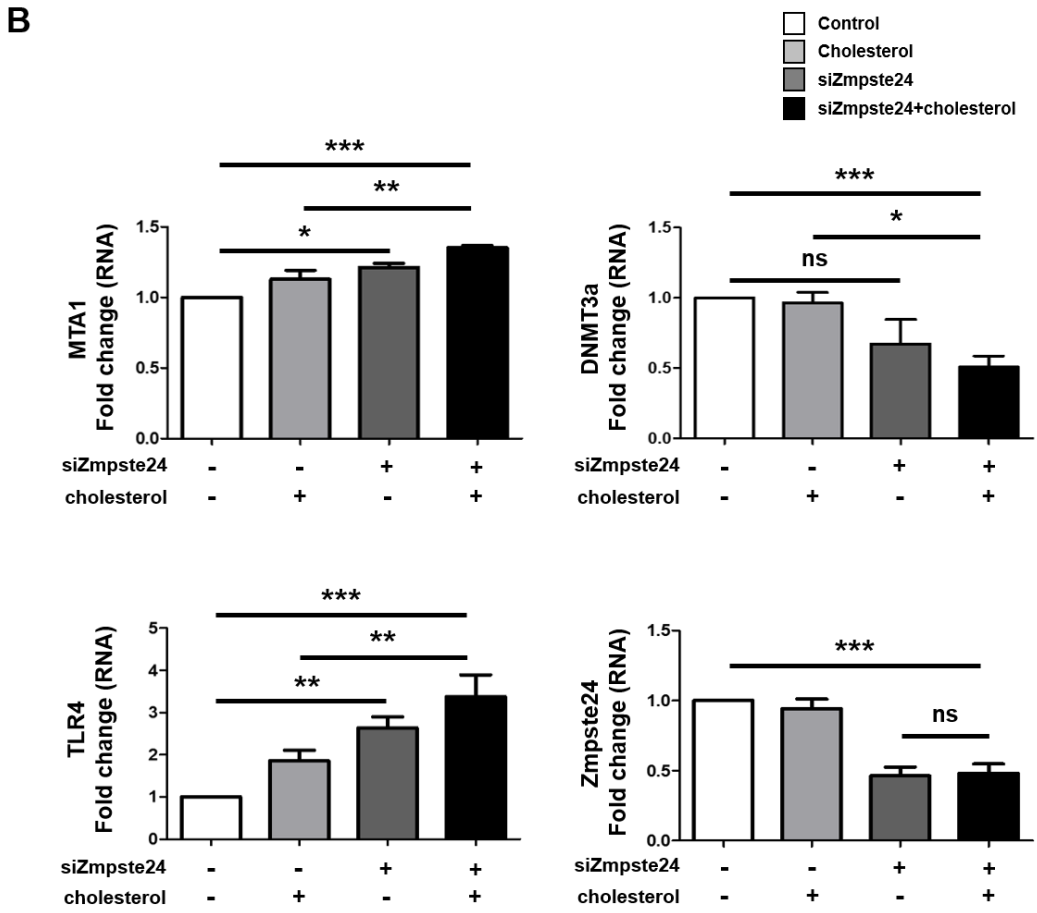


Figure 13. DNMT3a expression is reduced by MTA1 upregulation following Zmpste24 deficiency.

Vascular smooth muscle cells were pre-treated with Zmpste24 siRNA, and then cholesterol treatment for 48 hours.

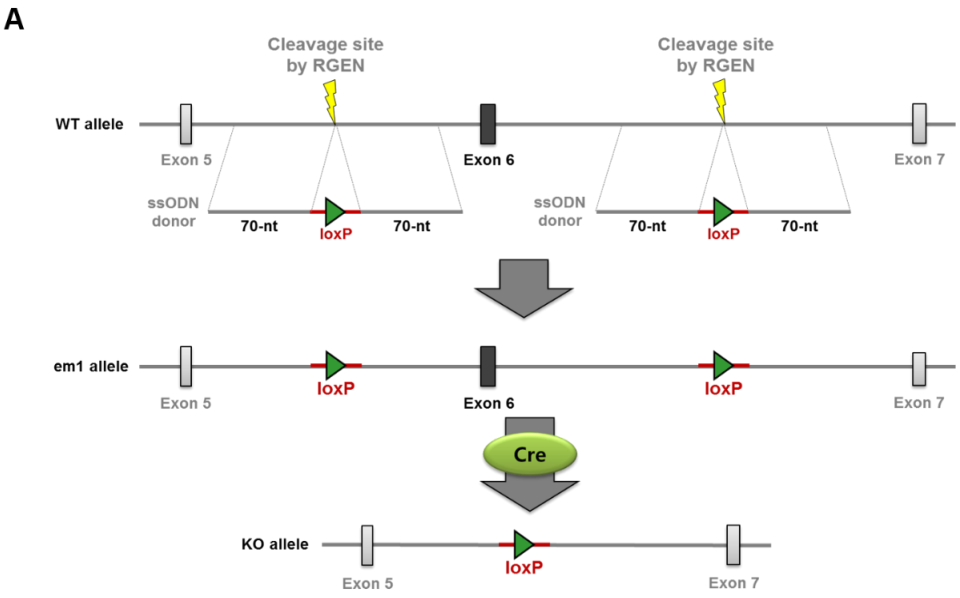
(A and B) The expression level of MTA1, DNMT3a, TLR4, Zmpste24 were assessed by RT-PCR. The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$, ***, $p < 0.001$; ns, not significant.

Part IV. An in vivo experimental model in mouse

13. Zmpste24 $^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice *in vivo* model

The experiment was carried out by receiving a transgenic mouse lines expressing a tamoxifen-activated Cre recombinase, CreER^{T2}, under the control of the smooth muscle-specific SM22 promoter. Zmpste24$^{F/F}$ mice with loxP cleavage sites inserted to the left and right of Zmpste24 gene exon6 were crossed with SM22αCre^{+} mice to generate Zmpste24 $^{F/F}$ SM22αCre^{+} mice. Next, Zmpste24 $^{F/F}$ SM22αCre^{+} mice and ApoE$^{-/-}$ mice were crossed with mice to finally generate Zmpste24 $^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice. The SM22α promoter allows VSMC-specific Zmpste24 deletion through Cre-loxP technology. When Tamoxifen was administered to this mouse, Cre released exon 6 to produce a mouse in which Zmpste24 was knockout specifically for SMC, and Zmpste24 expression test was conducted using these mice. On day 120, the mice were administered tamoxifen for 5 days and sacrificed 2 weeks later. To confirm that Zmpste24 was specifically knocked out in SMCs, we compared the SMCs isolated from aorta with tissues from other regions to see the expression of Zmpste24 at the RNA and protein levels. As a result, the expression of Zmpste24 did not differ between wild type and knockout mice in heart, kidney, liver, brain, lung, etc., but in the case of SMC, the significant lowering of the expression of Zmpste24 in the knockout mice compared to wild type mice was confirmed. In addition, since it is known that nuclear

abnormality occurs when Zmpste24 function is abnormal, we proceeded with Lamin A IF to check the morphology of the nucleus. While the morphology of the nucleus in other tissues was normal in both wild type and knockout mice, the nuclear in the Aorta's SMC showed blebbing of the nucleus in the knockout mice, confirming the SMC-specific Zmpste24 knockout.



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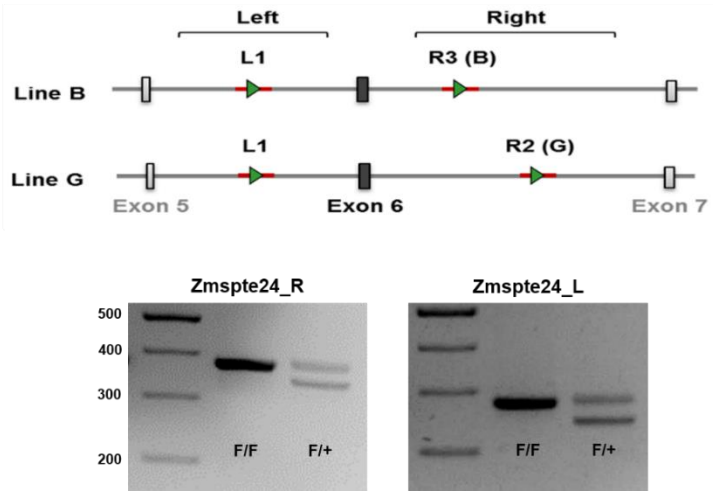

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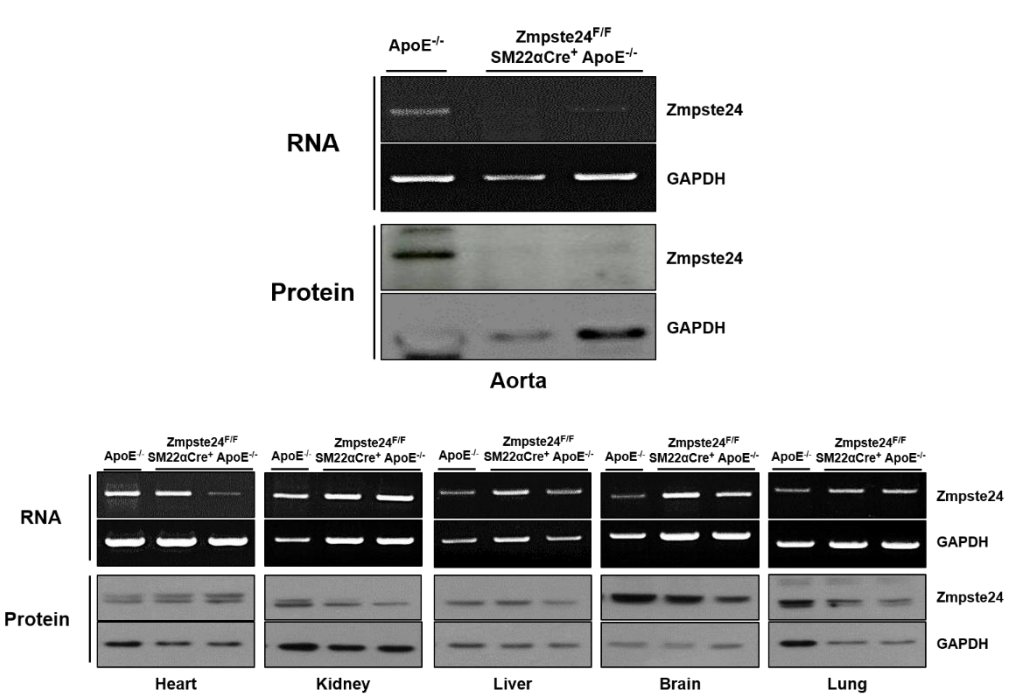


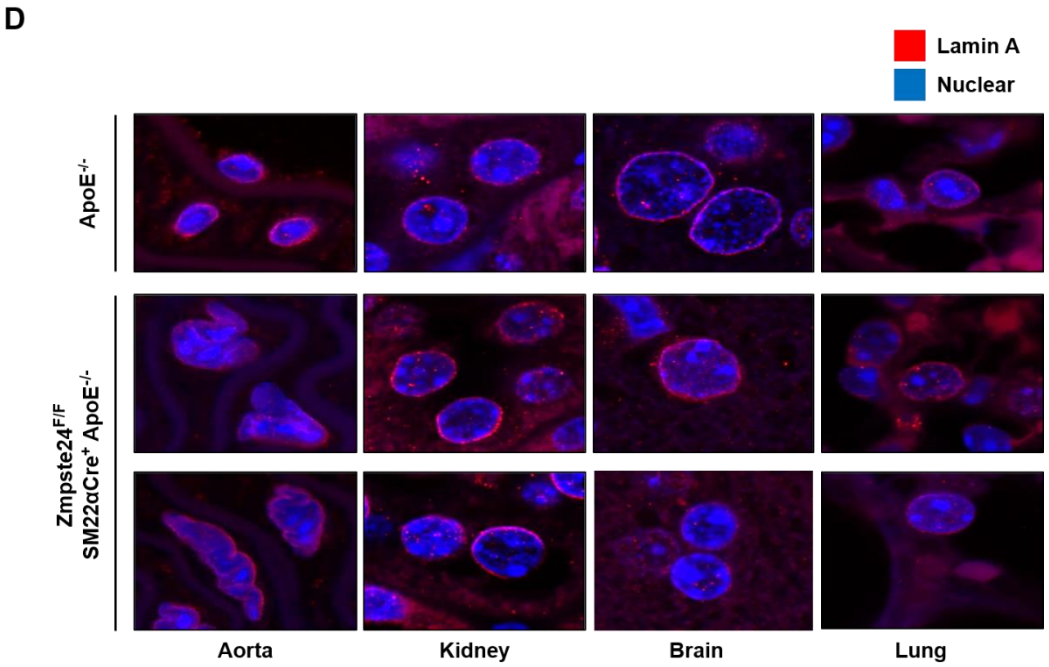


Figure 14. Characterization of Zmpste24 deletion in Zmpste24$^{F/F}$ SM22αCre^{+} mice.

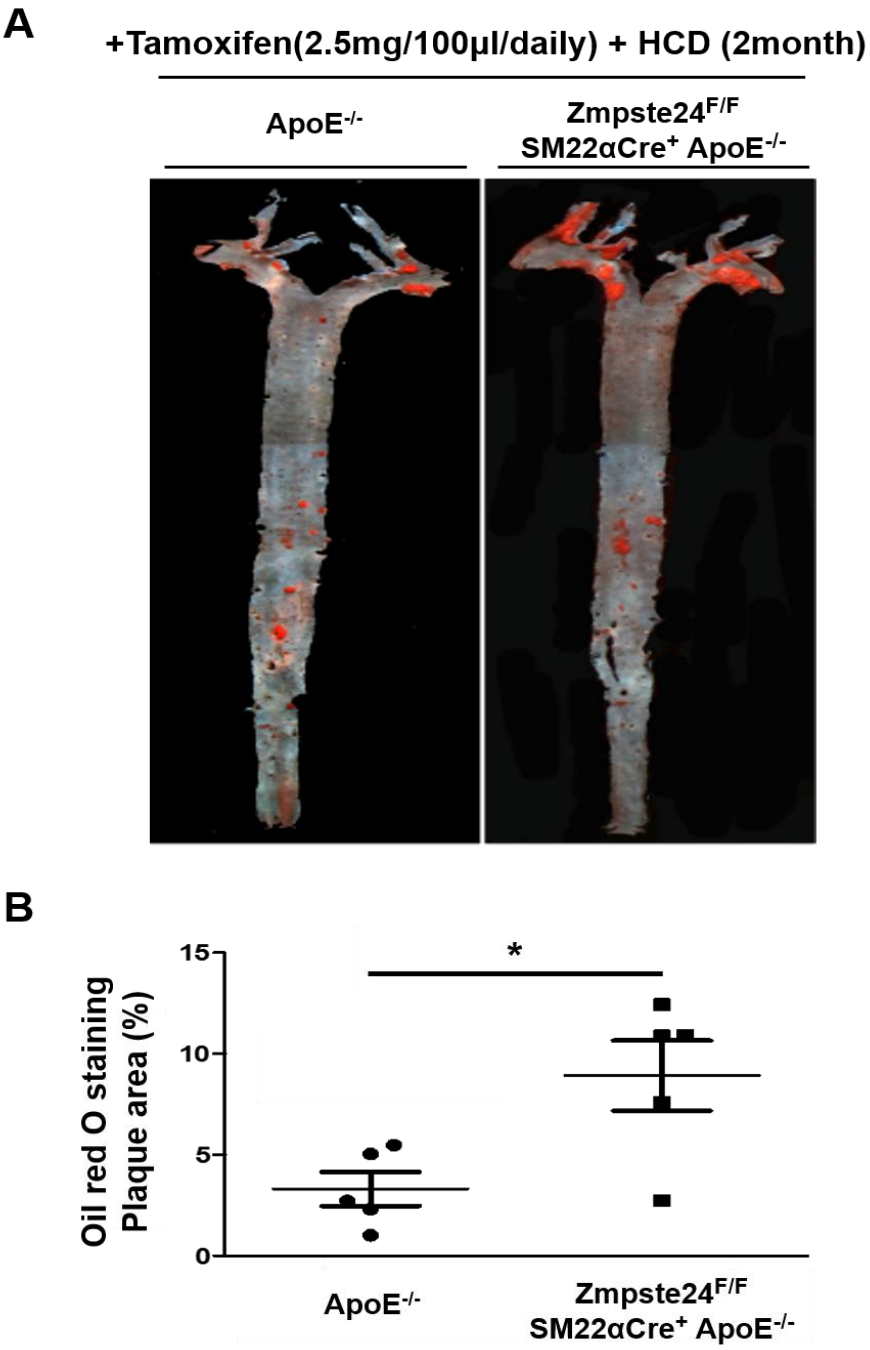
After intraperitoneal injection administration of tamoxifen to Zmpste24$^{F/F}$ SM22αCre^{+} mice, the Cre protein generated recognized the loxp site and Zmpste24 DNA exon 6 was removed, thereby deleting Zmpste24 from SMC. Zmpste24 expression test was performed using these mice.

(A) Schematic representation of smooth muscle cell-specific Zmpste24 knockout mice. (B) Genomic PCR identification of 120 days old Zmpste24$^{F/F}$ SM22αCre^{+}, Zmpste24$^{F/+}$ SM22αCre^{+} mice. (C) The expression level of Zmpste24 was assessed by RT-PCR and

western blot in isolated SMC from mice aorta. The expression level of Zmpste24 was assessed by RT-PCR and western blot in heart, kidney, liver, brain and lung. (D) Immunofluorescence analysis using staining for Lamin A (red) and nuclear (blue) from ApoE knockout mice and SMC-specific Zmpste24 knockout mice on aorta, kidney, brain and lung. Image of the tissue were photographed with confocal laser scanning microscope LSM780.

14. Smooth muscle22α-specific Zmpste24 deficiency promotes atherosclerotic plaque formation in ApoE$^{-/-}$ mice

8-week-old ApoE$^{-/-}$mice and Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice were intraperitoneal injection of total 25 μg Tamoxifen for 5 days and fed a high cholesterol diet for 8 weeks. After waiting for 8 weeks' diet period, the mice were sacrificed for obtained the aorta and aortic root, oil red O staining was performed to identify plaque areas in the aorta and aortic roots. As a result, it was found that plaque area significantly increased in the aorta and aortic root of Zmpste24 $^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$ mice. Therefore, it was found that smooth muscle22α-specific Zmpste24 deficiency accelerated atherosclerosis in ApoE$^{-/-}$ mice in vivo.



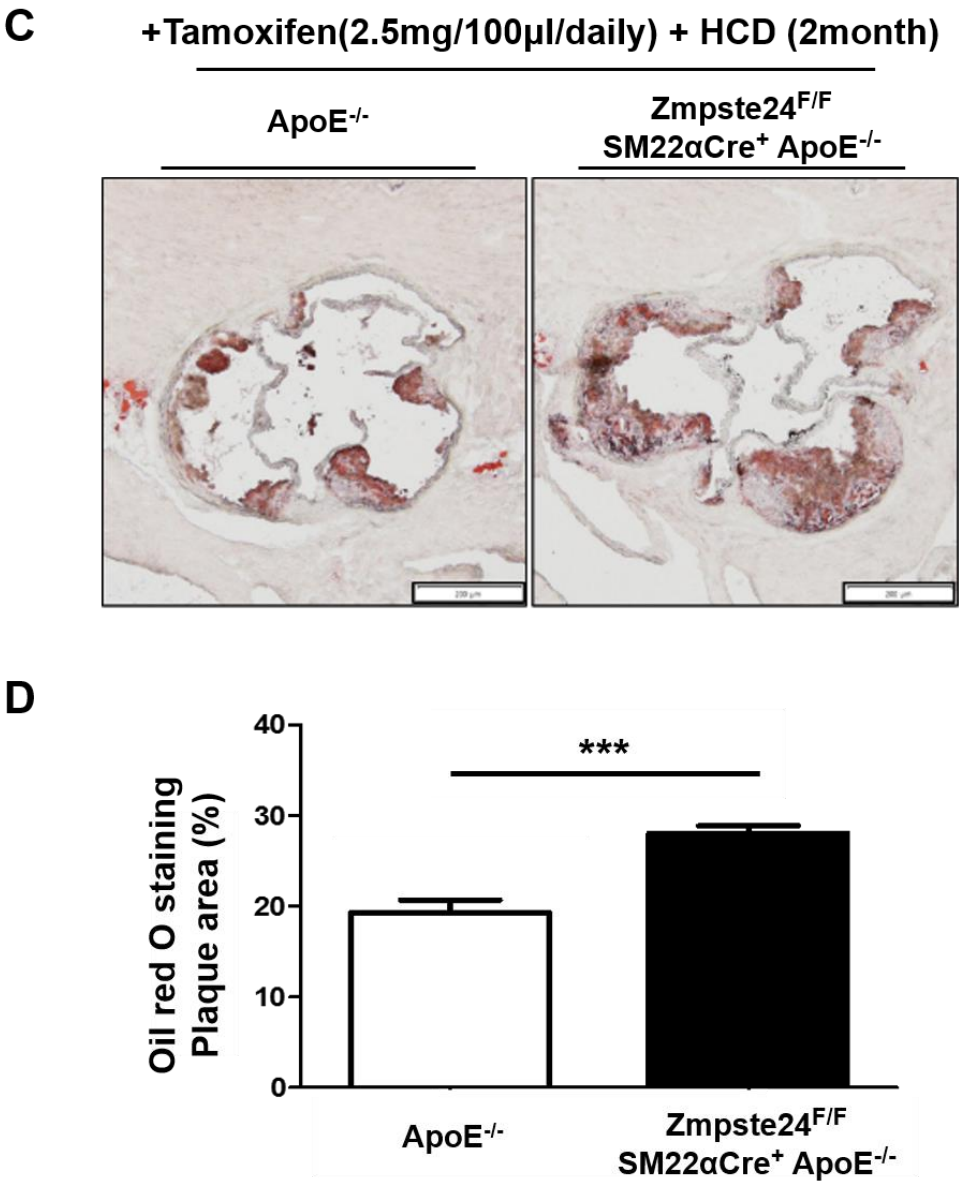


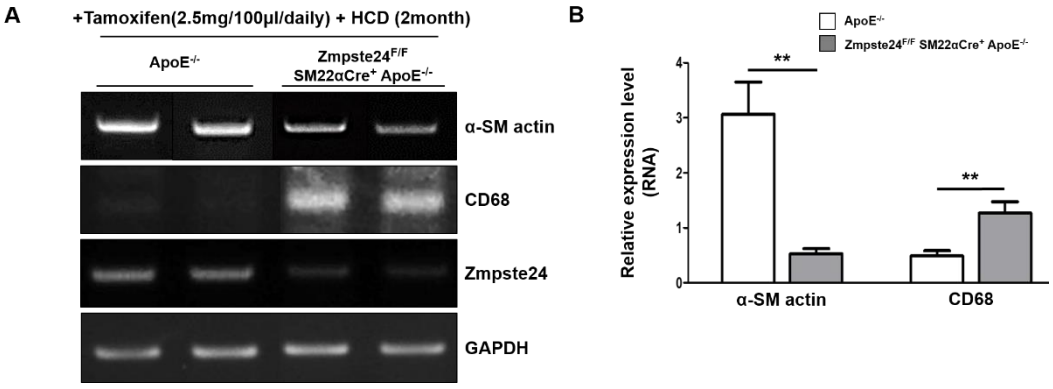
Figure 15. Smooth muscle22α-specific Zmpste24 deficiency increases atherosclerotic plaque area in ApoE$^{-/-}$ mice.

8-week-old ApoE$^{-/-}$mice and Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice were intraperitoneal injection of total 25 μg Tamoxifen for 5 days and fed a high cholesterol diet for 8 weeks.

(A) Representative images of consecutive en face aortic lesion areas stained with oil red after 8 weeks of high-cholesterol diet (HCD) in Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice. The graph represents the % of plaque area. (B) Representative images of consecutive aortic roots sections stained with oil red after 8 weeks of high-cholesterol diet (HCD) in Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice. The graph represents the % of plaque area. Values are mean ± SEM. *, $p < 0.05$; ***, $p < 0.001$

15. Smooth muscle22α-specific Zmpste24 deficiency promotes VSMC phenotypic switching in ApoE$^{-/-}$ mice

Next, I isolated SMC only from aorta to obtain RNA and protein to investigate the effect of Zmpste24 deficiency on VSMC phenotypic switching in vivo. In ApoE knockout mice, the expression of a-SMA was decreased and the expression of CD68 was increased by SMC-specific Zmpste24 deficiency. These results suggest that Zmpste24 deficiency accelerates VSMC phenotypic switching.



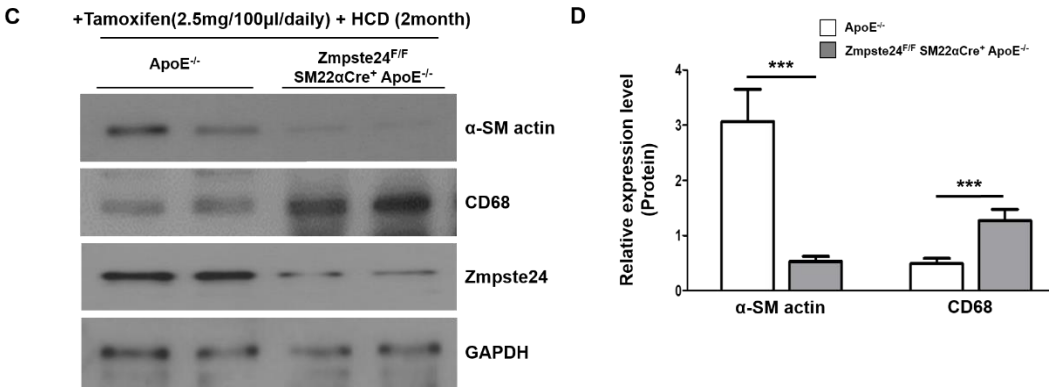


Figure 16. Smooth muscle22α-specific Zmpste24 accelerates VSMC phenotypic switching by regulating expression of a-SM actin, CD68 in ApoE$^{-/-}$ mice.

8-week-old ApoE$^{-/-}$mice and Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice were intraperitoneal injection of total 25 μg Tamoxifen for 5 days and fed a high cholesterol diet for 8 weeks (A) The expression of a-SM actin and CD68 were evaluated by RT-PCR in isolated SMC from mice aorta. The quantification was done by normalization of the density to that of GAPDH. (B) The expression of a-SM actin and CD68 were evaluated by western blot in isolated SMC from mice aorta. The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. **, $p < 0.01$, ***, $p < 0.001$

16. Smooth muscle22α-specific Zmpste24 deficiency in smooth muscle cells of ApoE$^{-/-}$ mice leads to increase TLR4 expression

Also investigated whether Zmpste24 deficiency increases TLR4 expression in VSMC in vivo. As was shown in vitro, it was confirmed that the expression of TLR4 was increased in SMC of SMC-specific Zmpste24 KO mouse.

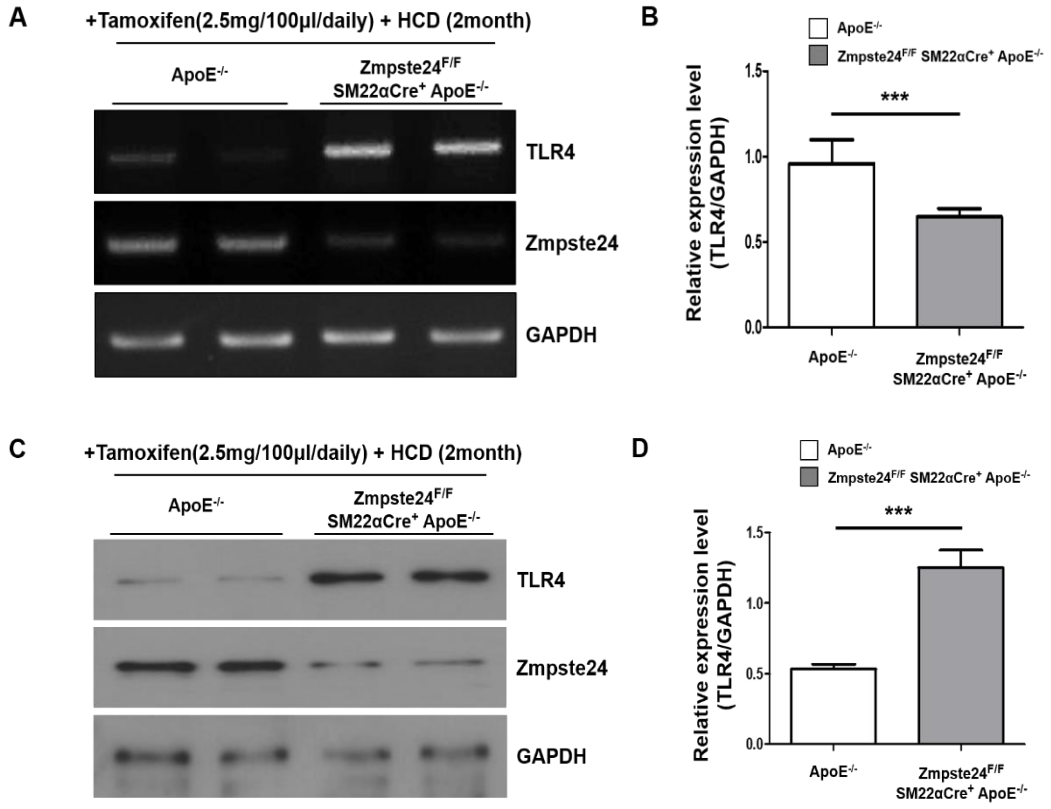


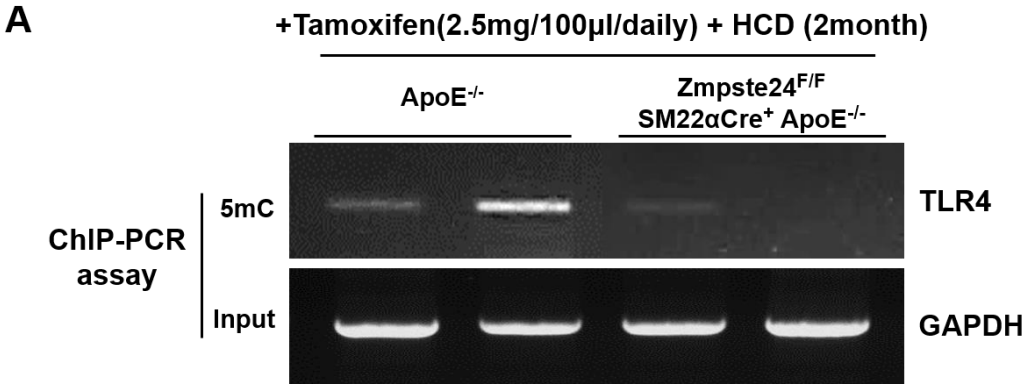
Figure 17. Isolated SMCs from Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice aorta showed increases expression of TLR4 expression compared to in ApoE$^{-/-}$ mice.

8-week-old ApoE$^{-/-}$mice and Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice were intraperitoneal injection of total 25 μg Tamoxifen for 5 days and fed a high cholesterol diet for 8 weeks.

(A and B) The expression of TLR4, Zmpste24 were evaluated by RT-PCR in isolated SMC from mice aorta. The quantification was done by normalization of the density to that of GAPDH. (C and D) The expression of TLR4, Zmpste24 were evaluated by western blot in isolated SMC from mice aorta. The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. ***, $p < 0.001$

17. Smooth muscle22α-specific Zmpste24 deficiency regulates TLR4 expression through DNA methylation

ChIP-PCR assay was performed to confirm whether the expression of TLR4 by Zmpste24 is regulated by DNA methylation as shown in vitro previously. Compared to ApoE KO, SMC-specific Zmpste24 KO showed a tendency to have decrease expression of methylated TLR4. In conclusion, even in vivo, SMC-specific Zmpste24 deficiency regulates TLR4 expression through DNA methylation.



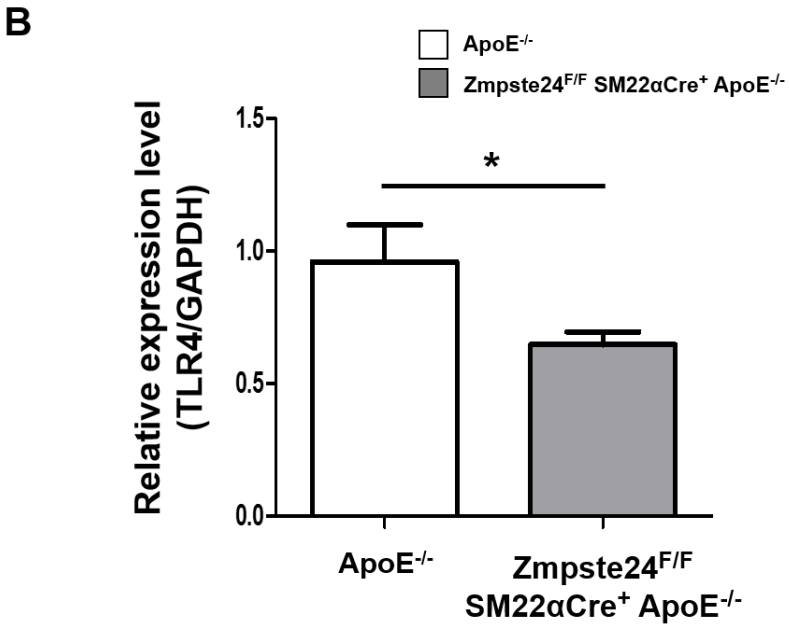


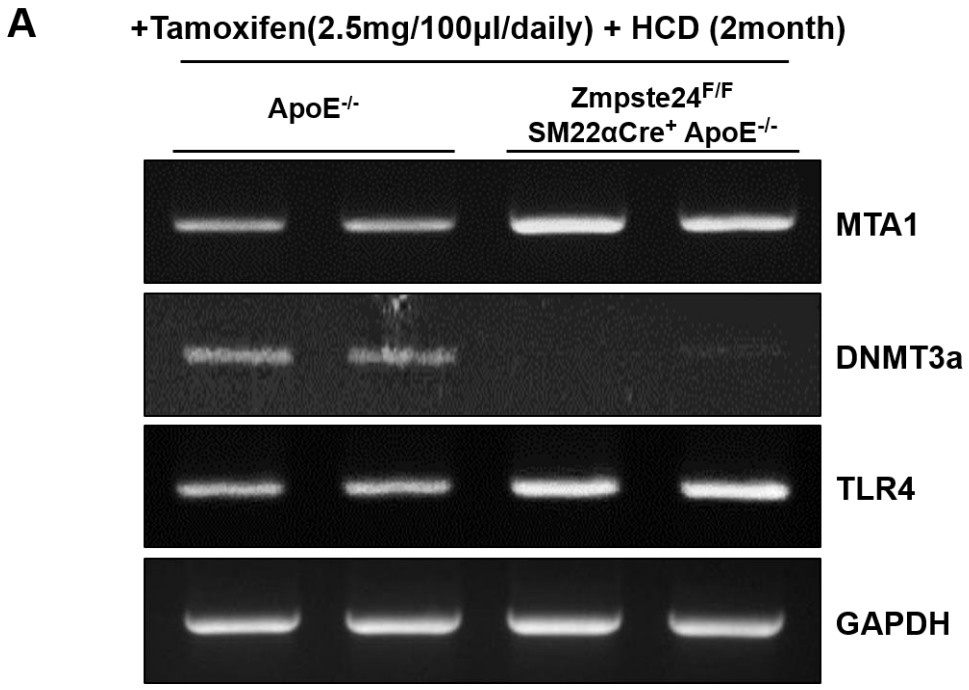
Figure 18. Smooth muscle22α-specific Zmpste24 Deficiency decrease methylated TLR4 expression in ApoE$^{-/-}$ mice.

8-week-old ApoE$^{-/-}$mice and Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice were intraperitoneal injection of total 25 μg Tamoxifen for 5 days and fed a high cholesterol diet for 8 weeks.

(A) Cross-linked cell lysates were subjected to ChIP-PCR analysis with anti-5mC antibody. RT-PCR was performed with ChIP primer as listed in Materials and Methods. The results present the means of five independent experiments. (B) The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$

18. Zmpste24 deficiency regulates atherosclerosis via MTA1-DNMT3a-TLR4 expression in ApoE$^{-/-}$ mice model

Next, I checked whether Zmpste24 is associated with changes in in the expression of MTA1-DNMT3a-TLR4 in vivo. As a result, we found that SMC-specific Zmpste24 deficiency in ApoE$^{-/-}$ mice was associated with increased expression of MTA1, decreased expression of DNMT3a and increased expression of TLR4, similar to the findings demonstrated in vitro.



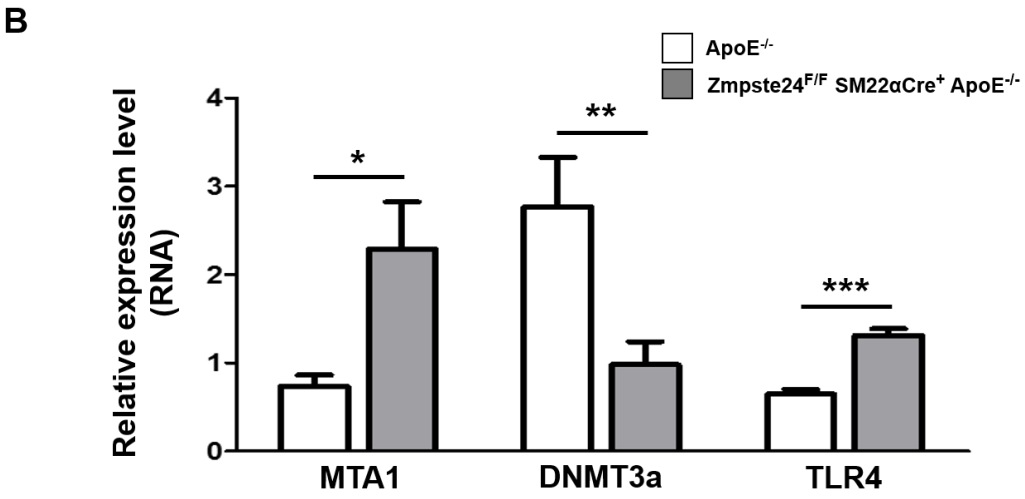


Figure 19. Smooth muscle22α-specific Zmpste24 Deficiency decrease DNMT3a expression by increasing MTA1 expression in ApoE$^{-/-}$ mice.

8-week-old ApoE$^{-/-}$mice and Zmpste24$^{F/F}$ SM22αCre^{+} ApoE$^{-/-}$ mice were intraperitoneal injection of total 25 μg Tamoxifen for 5 days and fed a high cholesterol diet for 8 weeks.

(A and B) The expression of MTA1, DNMT3a, TLR4 were evaluated by RT-PCR in isolated SMC from mice aorta. The quantification was done by normalization of the density to that of GAPDH. Values are mean ± SEM. **, $p < 0.01$

IV. DISCUSSION

The major findings of this study are as follows: First, Zmpste24 plays an important role in cholesterol-induced VSMC phenotypic switching and VSMC-foam cell formation in vitro and in vivo. Second, I confirmed that TLR4 plays an important role in VSMC-foam cell formation by showing that Zmpste24 deficiency is involved in cholesterol uptake through increased TLR4 expression. Third, I confirmed that Zmpste24 deficiency accelerates atherosclerosis by regulating TLR4 expression by epigenetic modification in vascular smooth muscle cells.

Modified LDLs, such as oxLDL and mmLDL, are the most common components of early arterial valves are involved in the pathogenesis of atherosclerosis. It has been reported that vascular smooth muscle cells exposed to modified LDL exhibit reduced expression of contractile markers and undergo phenotypic transition from vascular smooth muscle cells to macrophage-like cells [28]. Because there have been few studies of cholesterol-treated vascular atherosclerosis compared with modified LDL, I investigated the mechanism of cholesterol-induced vascular atherosclerosis. The study results confirmed that cholesterol treatment in VSMCs, like modified LDL treatment, reduced the expression of contractile markers in vascular smooth muscle cells and induced a phenotypic transition from vascular

smooth muscle cells to macrophage-like cells.

Zinc Metallopeptidase STE24 (Zmpste24) is an aging-related gene, and its main function is known as an enzyme that plays an important role in converting prelamin A into mature lamin A [3]. Zmpste24 deficiency or genetic defects cause laminates similar to lamin A defects. Zmpste24 function is known to be impaired during normal aging process and is associated with increase DNA damage, nuclear morphology defects, DNA repair system defects, and premature aging [4][5]. A previous study has shown that Zmpste24 total knockout mice have reduced lifespan due to accumulation of prelamin A in nuclear envelope, nuclear abnormalities, loss of subcutaneous fat, skeleton/muscular defects [6]. In Zmpste24-deficient mice, senescence is accelerated by the activation of p53 signaling [7], and prelamin A is said to accelerate VSMC senescence by inducing mitotic defects and DNA damage [6]. Taken together, there have been many studies indicating that lamin A processing abnormalities caused by defects in Zmpste24 accelerate aging; however, studies on other mechanisms are lacking. Also, although aging is the most important risk factor for atherosclerosis, it is difficult to study the role of aging of VSMCs on atherogenesis as whole genetic modified mice models would result in accelerated aging and decreased longevity. Therefore, I studied the effect of accelerate aging on atherosclerosis through conditional knockout of Zmpste24 of VSMCs in ApoE

knockout mice. In the present study, I demonstrated that deficiency of Zmpste24 in cholesterol-treated VSMCs reduces the expression of contractile markers in vascular smooth muscle cells, induces a phenotypic transition from vascular smooth muscle cells to macrophage-like cells, and promotes VSMC foam cell formation.

Cholesterol accumulates in macrophages or vascular smooth muscle cells through various mechanisms, such as various scavenger receptors, innate immunity receptors and endocytosis [17, 19]. I wanted to confirm the main mechanism by which cholesterol uptake was increased in VSMCs by cholesterol stimulation and Zmpste24 inhibition. First, to confirm whether endocytosis affects cholesterol uptake under these experimental conditions, the degree of VSMC foam cell formation was checked using Clatrin-, Caveolae-specific inhibitor. As a result, Clatrin- and Caveolae-mediated endocytosis did not affect cholesterol uptake under these experimental conditions. Next, LDLR, SR-A and CD36, scavenger receptors that are mainly responsible for lipid uptake in macrophages were screened by RT-PCR. However, they also did not show significant changes in vascular smooth muscle cells. Finally, increased expression of TLR4 in VSMCs stimulated with cholesterol was confirmed by RT-PCR and western blot with significantly increased expression of TLR4 when Zmpste24 siRNA and cholesterol were co-treated. As a result of treating Cholesterol-treated VSMC with TAK-242, a TLR4-specific

inhibitor, for each dose, it was confirmed that the foam cell formation decreased in a dose-dependent manner. Through this, it was clearly verified that TLR4 contributes to cholesterol uptake in VSMC. However, one limitation is that I do not know whether TLR4 directly mediates the uptake of cholesterol or stimulates some other unknown pathway to increase cholesterol uptake.

Next, in this study, I performed additional experiments focusing on Zmpste24 and TLR4 in cholesterol-treated VSMCs. Previous studies confirmed that TLR4 contributes to cholesterol uptake in VSMC, I tested the effect of TLR4 on cholesterol uptake in Zmpste24 deficiency using TAK-242. Our results showed that VSMC foam cell formation was further increased when Zmpste24 siRNA and cholesterol were treated together than cholesterol alone, and VSMC foam cell formation was significantly reduced in both groups when TAK-242 was pretreated. These results supported the notion that increased TLR4 expression plays an important role in VSMC foam cell formation when Zmpste24 is deficient.

Epigenetics is the study of genetic phenotypic changes that do not involve changes in DNA sequence, which is known to regulate expression primarily by triggering DNA methylation to modulate gene silencing. Recently, many studies have been conducted in the context of epigenetics to understand the molecular mechanisms underlying SMC plasticity [20] [21], it has been reported that Lamin

A is involved in heterochromatin regulation even under normal conditions and the expression of DNA methyltransferase in atherosclerotic lesions has also been reported [22]. Based on these research results, I determined that Zmpste24 regulates TLR4 expression through DNA methylation. In this study, I then confirmed through an MSP assay that most TLR4 genes were methylated and their expression was suppressed in normal VSMCs. In addition, after treatment with 5-az, a DNA methyltransferase inhibitor, the expression of methylated TLR4 was decreased and the expression of unmethylated TLR4 was increased in normal VSMCs. As with 5-az, to determine whether Zmpste24 regulates TLR4 methylation, normal VSMCs were treated with Zmpste24 siRNA, the MSP assay was performed, and the same results were obtained as with 5-az treatment. Also confirmed through a ChIP-PCR assay that the expression of methylated TLR4 was decreased when Zmpste24 siRNA and cholesterol were co-administered compared with cholesterol treatment alone. Therefore, these findings indicate that the expression of TLR4 is regulated by DNA methylation.

In previous studies by Chen, K. C., et al., Hamaidia, M., et al., the expression of DNMT3a and DNMT3b were reduced in VSMCs stimulated to oxLDL. However, little is known regarding the effect of Zmpste24 on related intracellular signaling pathways following cholesterol treatment of VSMCs. Accordingly, I screened

DNMT subunits in cholesterol-treated VSMCs after Zmpste24 siRNA treatment. In cholesterol-treated VSMCs, Zmpste24 deficiency decreased DNMT3a expression, whereas Zmpste24 overexpression increased DNMT3a expression. Also, I demonstrated by RT-PCR analysis that the expression of MTA1 was increased, not only by cholesterol treatment alone, but also when Zmpste24 siRNA and cholesterol were administered simultaneously. However, one limitation to this study is that the association or signaling between MTA1 and DNMT3a in cholesterol-treated VSMCs following Zmpste24 siRNA treatment is not yet clear.

In the in vivo study using $Zmpste24^{F/F}$ $SM22\alpha Cre^{+}$ $ApoE^{-/-}$ mice aorta, atherosclerotic plaque lesions were significantly increased compared to ApoE knockout mice. Also, the expression of MTA1/TLR4 was increased the expression of DNMT3a was decrease and TLR4 methylation was decreased in SMCs isolated from $Zmpste24^{F/F}$ $SM22\alpha Cre^{+}$ $ApoE^{-/-}$ mice aorta, confirming these findings from the in vitro experiment.

In summary, the present study shows that Zmpste24 deficiency in cholesterol-treated VSMCs significantly accelerates atherosclerosis in vitro and in vivo. In this study, I found that Zmpste24 regulates cholesterol-induced VSMC phenotypic switching and foam cell formation. Also, Zmpste24 deficiency was associated with significant increase in TLR4 expression in cholesterol-induced VSMCs. These

results suggest that Zmpste24 represents a promising therapeutic target for atherosclerosis protection in cardiovascular diseases.

V. CONCLUSION

In conclusion, the results provided evidence that Zmpste24 deficiency is associated with cholesterol-induced VSMC phenotypic switching and foam cell formation, which may be one of the underlying mechanisms for the acceleration of atherosclerosis. This study also demonstrated that Zmpste24 deficiency contributes to atherosclerosis by promoting cholesterol accumulation by regulating TLR4 expression through DNA methylation, one of the epigenetic modifications.

REFERENCES

1. Bäck, M., et al., *Inflammation and its resolution in atherosclerosis: mediators and therapeutic opportunities.* Nat Rev Cardiol, 2019. **16**(7): p. 389-406.
2. Basatemur, G.L., et al., *Vascular smooth muscle cells in atherosclerosis.* Nat Rev Cardiol, 2019. **16**(12): p. 727-744.
3. Young, S.G., L.G. Fong, and S. Michaelis, *Thematic Review Series: Lipid Posttranslational Modifications. Prelamin A, Zmpste24, misshapen cell nuclei, and progeria—new evidence suggesting that protein farnesylation could be important for disease pathogenesis.* Journal of Lipid Research, 2005. **46**(12): p. 2531-2558.
4. Varela, I., et al., *Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation.* Nature, 2005. **437**(7058): p. 564-568.
5. Liu, B., et al., *Genomic instability in laminopathy-based premature aging.* Nature Medicine, 2005. **11**(7): p. 780-785.
6. Afonso, P., et al., *LMNA mutations resulting in lipodystrophy and HIV protease inhibitors trigger vascular smooth muscle cell senescence and calcification: Role of ZMPSTE24 downregulation.* Atherosclerosis, 2016. **245**: p. 200-11.
7. Ragnauth, C.D., et al., *Prelamin A acts to accelerate smooth muscle cell senescence and is a novel biomarker of human vascular aging.* Circulation, 2010. **121**(20): p. 2200-10.
8. Liu, B., et al., *Depleting the methyltransferase Suv39h1 improves DNA repair and extends lifespan in a progeria mouse model.* Nat Commun, 2013. **4**: p. 1868.
9. Pendás, A.M., et al., *Defective prelamin A processing and muscular and adipocyte alterations in Zmpste24 metalloproteinase-deficient mice.* Nat Genet, 2002. **31**(1): p. 94-9.
10. Varela, I., et al., *Accelerated ageing in mice deficient in Zmpste24 protease is linked to p53 signalling activation.* Nature, 2005. **437**(7058): p. 564-8.
11. Hamczyk, M.R., et al., *Vascular Smooth Muscle-Specific Progerin Expression Accelerates Atherosclerosis and Death in a Mouse Model of Hutchinson-Gilford Progeria Syndrome.* Circulation, 2018. **138**(3): p. 266-282.
12. North, B.J. and D.A. Sinclair, *The intersection between aging and cardiovascular disease.* Circ Res, 2012. **110**(8): p. 1097-108.
13. Minamino, T. and I. Komuro, *Vascular aging: insights from studies on cellular senescence, stem cell aging, and progeroid syndromes.* Nat Clin Pract Cardiovasc Med, 2008. **5**(10): p. 637-48.
14. Shumaker, D.K., et al., *Mutant nuclear lamin A leads to progressive alterations of epigenetic control in premature aging.* Proc Natl Acad Sci U S A, 2006. **103**(23): p. 8703-8.

15. Gomez, D. and G.K. Owens, *Smooth muscle cell phenotypic switching in atherosclerosis.* Cardiovasc Res, 2012. **95**(2): p. 156-64.
16. Dubland, J.A. and G.A. Francis, *Lysosomal acid lipase: at the crossroads of normal and atherogenic cholesterol metabolism.* Front Cell Dev Biol, 2015. **3**: p. 3.
17. Moore, K.J., F.J. Sheedy, and E.A. Fisher, *Macrophages in atherosclerosis: a dynamic balance.* Nat Rev Immunol, 2013. **13**(10): p. 709-21.
18. Choi, S.H., et al., *Lipoprotein accumulation in macrophages via toll-like receptor-4-dependent fluid phase uptake.* Circ Res, 2009. **104**(12): p. 1355-63.
19. Wang, J.G. and M. Aikawa, *Toll-like receptors and Src-family kinases in atherosclerosis -- focus on macrophages.* Circ J, 2015. **79**(11): p. 2332-4.
20. Liu, R., K.L. Leslie, and K.A. Martin, *Epigenetic regulation of smooth muscle cell plasticity.* Biochim Biophys Acta, 2015. **1849**(4): p. 448-53.
21. Sen, P., et al., *Epigenetic Mechanisms of Longevity and Aging.* Cell, 2016. **166**(4): p. 822-839.
22. Greißel, A., et al., *Alternation of histone and DNA methylation in human atherosclerotic carotid plaques.* Thromb Haemost, 2015. **114**(2): p. 390-402.
23. Chen, K.C., et al., *OxLDL up-regulates microRNA-29b, leading to epigenetic modifications of MMP-2/MMP-9 genes: a novel mechanism for cardiovascular diseases.* Faseb j, 2011. **25**(5): p. 1718-28.
24. Hamaidia, M., et al., *Improvement of malignant pleural mesothelioma immunotherapy by epigenetic modulators.* Curr Top Med Chem, 2016. **16**(7): p. 777-87.
25. Deivendran, S., et al., *Metastasis-associated protein 1 is an upstream regulator of DNMT3a and stimulator of insulin-growth factor binding protein-3 in breast cancer.* Sci Rep, 2017. **7**: p. 44225.
26. Hsu, Y.F., et al., *p53 in trichostatin A induced C6 glioma cell death.* Biochim Biophys Acta, 2011. **1810**(5): p. 504-13.
27. Lantinga-van Leeuwen, I.S., et al., *Transgenic mice expressing tamoxifen-inducible Cre for somatic gene modification in renal epithelial cells.* Genesis, 2006. **44**(5): p. 225-32.
28. Chattopadhyay, A., et al., *Cholesterol-Induced Phenotypic Modulation of Smooth Muscle Cells to Macrophage/Fibroblast-like Cells Is Driven by an Unfolded Protein Response.* Arterioscler Thromb Vasc Biol, 2021. **41**(1): p. 302-316.

ABSTRACT (IN KOREAN)

혈관평활근세포에서 Zmpste24의 결핍은 후성유전학적 변형을 통한 TLR4의 발현을 증가시킴으로써 죽상동맥경화증을 가속화한다.

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조소영

목적: 죽상 동맥 경화증은 진행성 만성 염증성 질환이며 전 세계적으로 사망의 주요 원인으로 알려져 있다. 고령화는 동맥경화성 심혈관 질환의 주요 위험 요소이다. 그러나 이 현상의 근간이 되는 메커니즘은 여전히 불분명한 상태이다. 이 연구의 목적은 혈관 평활근 세포(VSMC) 표현형 전환 및 동맥경화 촉진에 대한 혈관 노화 관련-Zmpste24 유전자의 영향을 조사하여 근본적인 메

커니즘을 설명하는 것이다.

방법: 쥐 전체 대동맥에서 분리된 혈관 평활근 세포가 이 연구에서 사용되었다. Zmpste24 siRNA와 콜레스테롤 20μg/ml를 무혈청 DMEM에서 48시간 동안 처리하였다. VSMC의 콜레스테롤 축적은 오일 레드 O 염색으로 시각화 되었다. 표현형 전환 및 신호 전달 경로와 관련된 RNA 및 단백질의 발현을 RT-PCR 및 웨스턴 블롯으로 분석하였다. 생체 내에서 Tamoxifen IP를 통한 SMC 특이적 Zmpste24 녹아웃 마우스는 8주 동안 죽상경화증을 유도하기 위해 고콜레스테롤 식이를 먹였다.

결과: 우리의 데이터에 따르면, 콜레스테롤이 시험관 내 모델에서 VSMC-거품 세포 형성을 증가시킨다는 것이 오일 레드-O 염색을 사용하여 확인되었다. 콜레스테롤은 SMC 마커 발현의 하향 조절과 대식세포 마커 발현의 상향 조절에 의해 VSMC 표현형 전환을 유도했다. 또한 Zmpste24의 녹다운은 VSMC-거품 세포 형성과 콜레스테롤-VSMC 표현형 전환을 유의하게 촉진했다. 반대로, Zmpste24의 과발현은 VSMC 거품 세포 형성을 감소시켰다. 동물 연구에 따르면 평활근 특이적 Zmpste24의 녹아웃은 고콜레스테롤 식단을 섭취한 쥐의 전체 대동맥에서 죽상동맥경화증을 가속화 시켰다. 시험관 내 모델과 마찬가지로

동물 모델에서도 콜레스테롤-VSMC 표현형 전환이 가속화됨을 확인하였다.

결론: 현재 연구는 첫째, Zmpste24 결핍이 콜레스테롤 유도 VSMC 거품 세포 형성 및 콜레스테롤-VSMC 표현형 전환을 효과적으로 증가시켜 죽상 동맥 경화증을 가속화한다고 제안한다. 둘째, Zmpste24 결핍은 VSMC에서 후성유전학적 변형을 통해 TLR4 발현을 조절하여 콜레스테롤 축적을 촉진함을 제시한다. 종합하면, 이러한 결과는 심혈관 질환에서 죽상동맥경화증 보호를 표적으로 하는 약물의 미래 개발에 대한 통찰력을 제공할 수 있음을 시사한다.

핵심 되는 말: 동맥경화증; Zmpste24; TLR4; DNMT3a; 후성유전학적 변형