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MOLECULAR REGULATION OF VASCULAR CALCIFICATION IN MURINE MODELS OF ATHEROSCLEROSIS

Shanshan Gao

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MOLECULAR REGULATION OF VASCULAR CALCIFICATION IN MURINE

MODELS OF ATHEROSCLEROSIS

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**MOLECULAR REGULATION OF VASCULAR CALCIFICATION IN MURINE
MODELS OF ATHEROSCLEROSIS**

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
MD Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment
of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

by

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Molecular Regulation on Vascular Calcification in Murine Models of Atherosclerosis

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Background: Calcification occurs often in the atherosclerotic lesions of patients with coronary heart disease and animals with hypercholesterolemia, such as apolipoprotein-E deficient (ApoE^{-/-}) mice. However, the mechanism(s) underlying the development of calcification in atherosclerosis remains unclear. ApoE acts as a lipid transporter, but also has been recognized as a potential regulator of osteogenesis. Little information is available as to whether ApoE has any direct impact on osteogenesis and calcification in vascular smooth muscle cells (VSMC). Several signal transduction pathways play a role in regulation of calcification, including the Wnt/ β -catenin system and potentially GTAP, an ubiquitin-conjugating enzyme responsible for protein degradation, which participates in the homeostasis of proteins and metabolites in stem cells. The long-term goal of this study is to delineate the molecular pathway(s) by which ApoE regulates expression of osteogenic genes and calcification in vascular VSMC of atherosclerotic lesions.

Hypothesis: ApoE functions as an inhibitor of vascular calcification, through regulation of the function of Wnt/ β -catenin and GTAP signaling pathways.

Methods and Results: ApoE^{-/-} and wild type control mice were used for histopathology and cell culture studies. Compared to the aortic tissue and VSMCs of wild type C57BL/6J mice, ApoE^{-/-} aortic tissue and VSMCs from ApoE^{-/-} mice showed markedly increased calcification, and enhanced expression of pro-osteogenic genes but lowered expressions of myogenic biomarkers. Incubation of VSMCs with native ApoE protein added to culture or ApoE cDNA delivery by plasmid transfection or lentiviral infection reduced the levels of osteogenic genes (e.g. Runx2, BMP-2 and ALP) as well as calcification in ApoE^{-/-} VSMCs exposed to elevated inorganic phosphate (Pi). The genetically modified VSMCs isolated from ApoE^{-/-} mice displayed increased activation of the canonical Wnt/ β -catenin pathway at the presence of Pi, which could be reversed by incubation with native ApoE protein in culture. The GTAP-deficient aortic rings developed more severe calcification when exposed to Pi. Moreover, the half-life of the osteogenesis-regulator Runx2 shortened in VSMCs with GTAP overexpression.

Conclusion: ApoE serves as a critical inhibitor of vascular calcification through the Wnt/ β -catenin pathway. This study provides direct evidence that ApoE regulates calcification by regulating expression of certain pro-osteogenic factors, such as Runx2, BMP2 and ALP. Besides, GTAP is identified as a novel regulator of osteogenesis by mediating Runx2 protein stability.

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Abbreviations and Terms

α SMA: Alpha-smooth muscle actin

ALP: Alkaline phosphatase

ApoE: Apolipoprotein E

BMP-2: Bone morphogenetic protein 2

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GTAP: Galactosyltransferase-associated protein

LRP: Lipoprotein receptor-related protein

OPN : Osteopontin

OSC: Osteocalcin

Runx2: Runt-related transcription factor 2

SM22 α : Smooth muscle 22 alpha

UBC: Ubiquitin binding core

VLDL: Very low density lipoprotein

VC: Vascular Calcification

VSMC: Vascular smooth muscle cells

Chapter 1 : Background

1. Atherosclerosis and Vascular Calcification

1.1 Vascular calcification in the arterial tissues and atherosclerosis

Calcification is a common histopathological condition seen in aging or diseased organs and tissues, particularly arteries with atherosclerosis and other disorders which can cause vascular tissue injury. It frequently occurs in the arterial tissues of people over 55-65 years old, and may represent a hallmark of certain types of atherosclerotic lesions, especially those with active vascular cell proliferation and inflammation[1]. In the early phase of atherosclerosis, the lesions are largely restricted to the intima and the media of the arteries [2, 3]. During vascular calcification, calcium mineral deposition accumulates in and eventually stiffens arteries, causing substantial morbidity, including hypertension, myocardial ischemia and congestive heart failure[4].

The development of vascular calcification resembles osteogenesis, in which non-calcifying cells are induced to express osteogenic genes, and attract calcium to form insoluble deposits. Normally, contractile vascular smooth muscle cells (VSMCs) express low or no osteogenic genes but under the stimulation of atherogenic factors, VSMCs transdifferentiate into osteoblast-like cells and undergo calcification[5]. Calcium deposition can be found in atherosclerotic plaques, and atherosclerotic calcification is usually accompanied by chronic inflammation [6]. Of note, vascular calcification in later stages is still considered irreversible nowadays due to the

insolubility of calcium deposits and, therefore, prevention and regulation of calcification in earlier stages are of great importance.

Atherosclerosis is characterized by plaque build-up inside tissues inside the arterial walls, which can narrow the lumen of the blood vessels and cause thrombosis if rupture happens. Large quantities of epidemiological investigations have revealed risk factors linked to atherosclerosis such as hyperlipidemia, diabetes mellitus, smoking, hypertension and aging [7]. Atherosclerotic plaque contains cholesterol, fatty substances and cellular waste products[8, 9]; rupture of plaques releases pro-thrombogenic and pro-coagulating tissue factors, which trigger formation of thrombi, subsequently disruption of the blood stream, and consequently ischemia of distal organs or tissues, namely the heart and brain[10]. Calcium deposits are frequently found in atherosclerotic lesions as well [11]. Calcification can start early and accelerate as atherosclerosis progresses.

1.2 The role of Apolipoprotein-E in vascular calcification

Apolipoprotein E (ApoE), 299 amino acids long, is a 34kDa protein that consists of two major domains: the lipid-binding domain and the receptor-binding domain. Multiple functions of ApoE have been identified, such as regulation of glucose metabolism [12], beta-amyloid aggregation (A β) and tangle formation [13, 14], but the best-known feature of ApoE is its capability of binding-lipid capability[15]. Characterized as a secreted protein, ApoE is mainly synthesized in the

liver and brain, but is also widely distributed in other organs. ApoE facilitates the transport and thus clearance of lipid in brain and the circulation system. There are three isoforms of ApoE: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158), which differ in amino acid(s) at positions 112 or/and 158. The variance of amino acids at these positions alters the structure and functions of ApoE[16, 17]. ApoE2 exhibits a lower-affinity binding to receptors in comparison to ApoE3 and ApoE4[18]. ApoE2 and ApoE3 preferentially bind to HDL while ApoE4 prefers VLDL [19]. ApoE2 is associated with risks of atherosclerosis [20], whereas ApoE4 has been implicated in Alzheimer's disease [21], impaired cognitive function [22], HIV[23] and atherosclerosis[15]. Lack of ApoE leads to abnormally elevated level of lipids called hyperlipidemia. Apolipoprotein-E knockout (ApoE^{-/-}) mice spontaneously develop atherosclerosis in an age-dependent manner [24]. C57BL/6 mice at a young age (5-8 weeks old) can be induced to develop vascular calcification with vitamin D injection in the absence of atherosclerosis [25].

ApoE^{-/-} mice tend to have more severe atherosclerosis and calcification compared to the background strain, partially due to increased blood lipids. LDL receptors and LDL receptor-related protein-mediated clearance of remnant lipoprotein maintains the normal cholesterol level in plasma. The clearance of cholesterol-rich lipoprotein remnant in ApoE^{-/-} mice is compromised, which leads to accumulation of lipid deposits in the arterial walls, which develop into atherosclerotic plaques as it progresses [24]. Beyond lipid metabolism, ApoE acts as a suppressor of inflammation as well. It was noted that ApoE^{-/-} mice are prone to endotoxemia and bacterial

infection [26, 27]. Under most circumstances, ApoE functions via receptor binding. Currently there are 7 identified receptors that recognize ApoE as a ligand, all of which belong to the evolutionarily conserved low-density lipoprotein receptor (LDLR) family [28]. Among them, low density lipoprotein receptor (LDLR), low density lipoprotein receptor related protein 1 (LRP1), low density lipoprotein receptor related protein 5 (LRP5), low density lipoprotein receptor related protein 6 (LRP6), very low density lipoprotein receptor (VLDLR) and apolipoprotein E receptor 2 (ApoER2) are expressed in VSMCs [29-31].

Studies have explored the relationship between ApoE and osteoblast differentiation, and showed that ApoE negatively regulates osteogenesis. Interestingly, ApoE^{-/-} mice were found to have increased bone mass and bone mineral density [32], but the mechanisms by which ApoE regulates bone formation are not well understood. Moreover, ApoE appears to be regulated by BMP-2 [33] and expressed in the later stages of osteoblast differentiation [34].

1.3 Canonical Wnt/ β -catenin pathway and vascular calcification

The Wnt proteins are a family of secreted glycoproteins that bind to Frizzled family receptor and various coreceptor(s) depending on which Wnt pathway is activated [35]. Noncanonical pathways refer to Wnt signaling pathways that do not involve β -catenin and canonical pathway involves β -catenin [36]. When Wnt/ β -catenin signalling is inactive, β -catenin in cytoplasm is constantly degraded by a destruction complex which contains Axin, adenomatosis polyposis coli (APC) and glycogen

synthase kinase 3 (GSK3). This destruction complex prevents β -catenin accumulation in cytoplasm. Binding of Wnt ligand(s) to Frizzled and LRP5/6 coreceptor recruits Axin to the cytoplasmic part of receptor and disrupt the destruction complex. In this case, β -catenin accumulates in cytoplasm and eventually translocates into the nucleus, activating transcription of downstream genes [37]. Canonical Wnt/ β -catenin pathway plays a pivotal role in diverse physiological processes such as cell differentiation [38], cell proliferation [39] and cell-cell adhesion [40]. Wnt/ β -catenin pathway plays an indispensable part in vascular calcification. *In vitro* studies showed Wnt/ β -catenin pathway is activated when valvular interstitial cells differentiate into osteoblast-like cells [41]. Wnt/ β -catenin is also active in several osteoblast or osteoblast precursor cell lines including C3H10T1/2 cells and C2C12 cells[42], which implicates its importance in the regulation of osteogenesis.

As a response to a calcification-inducing stimulus such as high phosphorus levels, the β -catenin pathway becomes activated, and increased nuclear β -catenin acts as a transcription factor that leads to enhanced expression of downstream osteogenic genes, including osteopontin(OPN) and osteocalcin(OCN), resulting in enhanced calcification[43].

2. Phenotypical Transformation of Vascular Smooth Muscle Cells in Atherosclerosis and Calcification

2.1 Histology of blood vessels

The arteries and veins consist of three concentric layers: intima, media and adventitia[44]. The intima comprises a single layer of endothelial cells and a small amount of connective tissues. The media is the thickest layer of blood vessel walls, which provides structural support. The media contains vascular smooth muscle cells (VSMCs), the relaxation and contraction of which determine the elasticity and diameter of vessels. The adventitia is mainly composed of connective tissue and fibroblasts.

2.2 Contributions of cellular components to calcification

Once considered as a passive degeneration, vascular calcification turns out to be an actively regulated process that involves multiple cellular players[4]. Lineage tracing studies showed vascular endothelial cells might serve as a source of osioteoprogenitor cells and secrete a high level of BMP-2 under the stimulation of TNF- α , thus promoting osteogenic differentiation of VSMCs[45]. Endothelial cells are also capable of mediating calcification via formation of microparticles[46]. Fibroblasts, when treated with TGF- β 1 and elastin degeneration products, are capable of differentiating into osteoblast-like cells [47]. However, so far there are few reports that use cultured endothelial cells or fibroblasts to study calcification, possibly due to the lack of a reproducible and stable way to induce calcification in these two types of cells.

2.3 Induction of VSMC calcification under disease conditions

Vascular calcification might happen under conditions other than atherosclerosis, such like chronic kidney disease (CKD) [48]. Vascular calcification is a common complication in patients with CKD, attributed to an abnormally elevated phosphate level in blood. *Jono et al.* found VSMCs treated with increased concentrations of inorganic phosphate (Pi) in culture became calcified, which is dependent on a sodium-phosphate cotransporter named Pit-1[49]. Since then, VSMCs under the stimulus of Pi have been used as an *in vitro* model to study calcification. Mature VSMCs express various types of contractile proteins that are important for their function. As the main cellular component and structural support of the vessel wall, VSMCs control elasticity of major arteries, while calcified VSMCs greatly jeopardize the integrity of blood vessels. VSMCs exhibit extensive phenotypic plasticity and undergo phenotypic switching during development or in response to environmental cues [50]. When calcification happens, VSMCs gradually lose their contractility, and transdifferentiating into osteoblast-like cells. The smooth muscle lineage markers smooth muscle α -actin (α SMA) and smooth muscle 22 α (SM22 α) decrease in this process; calcification markers like Runx2, alkaline phosphatase (ALP) and osteopontin (OPN) are increased.

Besides, calcification can be initiated by membrane bound matrix vesicles (MVs) produced by living VSMCs and increased by apoptotic bodies released from

dying VSMCs. MVs secreted by VSMCs stimulated with calcification-causing factors contain preformed calcium deposit and calcify extensively[51]. Apoptosis usually precedes calcification and apoptosis of VSMCs has been shown to promote medial degeneration and calcification. Apoptotic bodies released by VSMCs act as nucleation sites for mineralization and concentrate calcium [52].

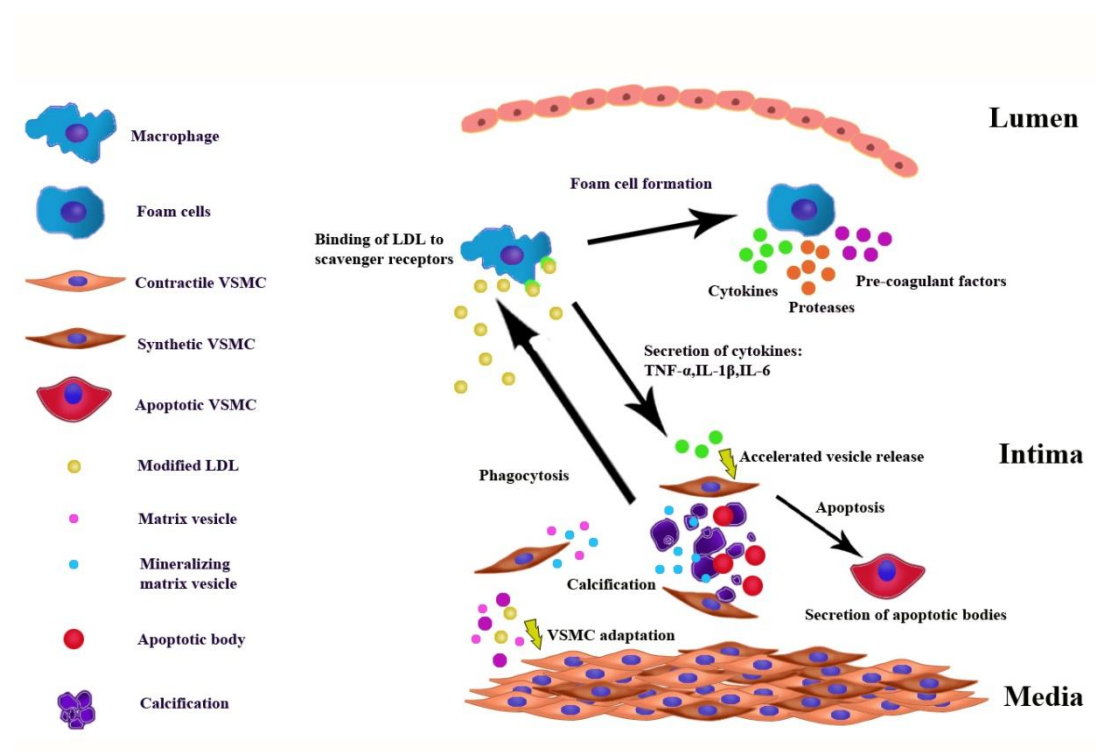


Figure 1: Inflammation and other pathogenic processes occurring in atherosclerotic calcification *in vivo*. Activated macrophages secrete cytokines such as $TNF-\alpha$, $IL-1\beta$ and $IL-6$, which stimulate vascular smooth muscle cells (VSMCs) to release more matrix vesicles, accelerating formation of calcium deposits inside the blood vessels. Apoptotic bodies secreted by apoptotic VSMCs as well as matrix vesicles act to exacerbate calcification. During atherosclerosis, VSMCs undergo phenotypic switch from contractile phenotype to synthetic phenotype.

Therefore, VSMCs are critical contributors to vascular calcification and inhibition of osteogenesis or apoptosis of VSMCs might be used as an effective way to regulate progression of calcification and maintain integrity of arteries.

2.4 Classification of calcification

There have been different opinions about the classification of calcifications. In general, there are at least 5 types of calcification identified so far: atherosclerotic calcification, medial calcification, calcific aortic valve disease, calcific uraemic arteriolopathy and the vascular calcification of end stage renal disease (ESRD), based on histoanatomic criteria[53]. In 1903, Johann Georg Mönckeberg described a type of calcification called Mönckeberg's arteriosclerosis, in which calcium deposits are found in the medial layer of blood vessels. It is still unclear whether Mönckeberg's arteriosclerosis is distinct from the five types of calcification mentioned above [54], but it might be complicated by atherosclerosis[55], diabetes[56] or some other disease. Since atherosclerosis is characterized by infiltration of lipid and macrophages into the arterial wall, atherosclerotic calcification invariably associates with these two elements. Besides, recruitment of VSMCs to the intima of blood vessels is common in advanced atherosclerotic lesions [57]. Therefore, although the calcium deposits in atherosclerotic plaques are often observed in the intima, it involves various types of cells other than intimal endothelial cells. Medial calcification, conversely, is almost

solely associated with VSMCs compared to intimal calcification.

3. The Ubiquitin-Proteasomal System for Regulation of Protein Homeostasis

3.1. Pathways of protein degradation

Protein turnover stands for the balance between protein synthesis and protein degradation, which is vital to the normal physiological functioning of cells. There are two major pathways mediating protein degradation in eukaryotic cells: the lysosomal proteolysis and ubiquitin-proteasome pathway. The lysosomal proteolysis involves uptake of proteins by lysosomes, which are organelles enclosed in membranes and containing digestive enzymes[58]. One major pathway for this uptake is autophagy, in which vesicles from endoplasmic reticulum fuse with lysosomes. The autophagy-lysosome pathway is normally non-selective. The ubiquitination-proteasome pathway, by contrast, appears to be more involved in selective and rapid degradation of proteins.

3.2. Ubiquitination

Ubiquitin, a small protein around 8.5 kDa, is universally expressed in almost all the tissues of eukaryotic organisms. It was discovered by Goldstein and his colleagues in 1975[59] and further studied by other scientists in 1970s and 1980s[60]. Ubiquitination refers to the process where one or more ubiquitin molecules are attached to protein residue(s), which affects the degradation, translocation or even

activity of target proteins. In terms of polyubiquitination-mediated protein degradation, there are three major enzymes involved: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). Polyubiquitination is initiated by E1 via an ATP-dependent reaction, resulting in formation of a thioester bond between the C-terminal carboxyl group of ubiquitin and the cysteine sulfhydryl group of E1 enzyme[61]. Then E2, the ubiquitin-conjugating enzyme binds to both the activated ubiquitin and E1, mediating the transfer of ubiquitin to the active cysteine residue at the C-terminus of E2 [62]. Afterwards, E3 the ubiquitin ligase, binds to E2 and substrate protein, creating an isopeptide bond between ubiquitin and the target protein. Repeating ubiquitination steps add additional ubiquitins to the substrate protein, forming a polyubiquitin chain[63]. Ubiquitinated proteins are recognized by the 19S complex of the proteasome and are then unfolded, and digested by the assembled 26S proteasome[64].

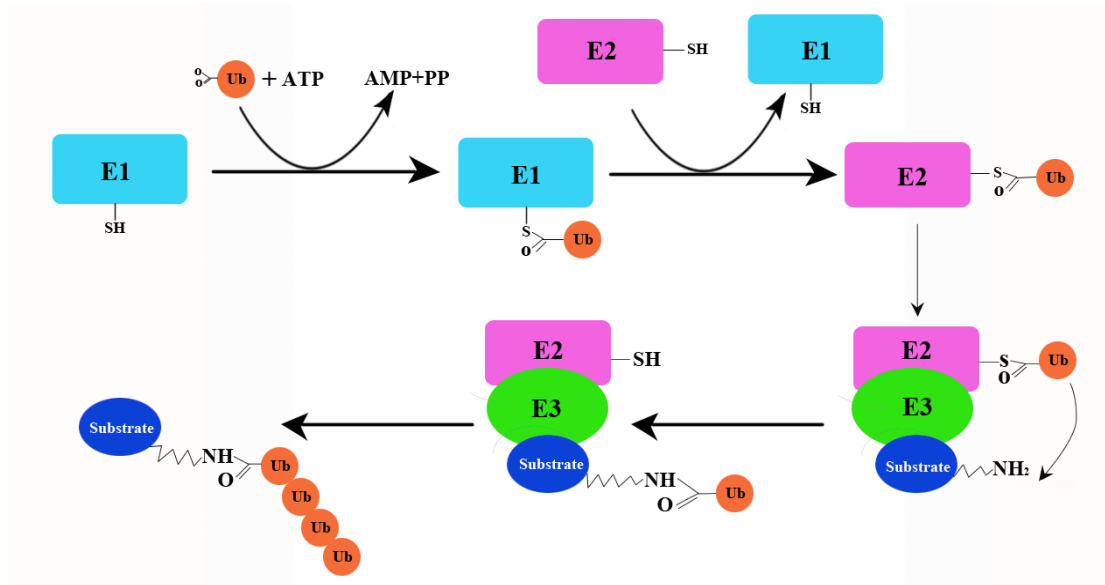


Figure 2. Ubiquitylation process via E1, E2 and E3 enzymes. The ubiquitin-activating enzyme (E1) binds to the C-terminus of ubiquitin and activates it, a process dependent on ATP hydrolysis. The activated ubiquitin is then transferred from E1 to the conserved cysteine residues of an ubiquitin-conjugating enzyme (E2). The E2 protein functions together with ubiquitin ligases(E3). E2-E3 complexes bind to protein substrates and mediate the transfer of ubiquitin to a lysine residue on the protein substrate. A multiubiquitin chain will be formed after this process is repeated. The multiubiquitin chain can be recognized by a specific receptor in the proteasome and subjected to degradation.

To date, two E1[65] and thirty-five E2 enzymes [66] have been identified from the human genome, which encodes hundreds of putative E3 ligases, granting enormous variety to protein substrates. Since ubiquitination regulates degradation of proteins, it is required to maintain the proteostasis in organisms. Disorders of ubiquitination lead to accumulation of misfolded proteins in the body, causing a plethora of diseases, including cancer, metabolic syndromes, inflammatory disorders and muscle dystrophies [67-69].

3.3 Galactosyltransferase 1-associated protein

All ubiquitin-conjugating (E2) enzymes contain a highly conserved ubiquitin-conjugating catalytic domain (UBC), which is usually 150-200 amino acids long [70]. E2s are classified according to the existence of extensions to the catalytic domain. Class I E2s only contain the catalytic domain, whereas class II, III and IV E2s have N- or/and C- terminus extensions. These extensions determine the functional differences between E2s [66]. Galactosyltransferase 1-associated protein (GTAP), also known as Ube2q1, is a member of the class III ubiquitin-conjugating enzyme family. The Ube2q family has several members, in addition to GTAP, which all consist of a unique N-terminus and a RWD domain.

GTAP has been shown to interact with and regulates the subcellular localization of galactosyltransferase 1 (GalT1), a protein that potentially modulates

stem cell pluripotency and differentiation. GTAP is ubiquitously expressed in all organs and particularly highly expressed in lung, kidney and reproductive organs [71]. Embryonic stem cells have a potent expression of GTAP as well, while differentiated cells such as endothelial cells and smooth muscle cells express a relatively low level of GTAP.

Chapter 2 : Hypotheses and Specific Aims

1. Impact of ApoE on atherosclerosis and vascular calcification

Because atherosclerotic plaques frequently become calcified, calcification has been used as an alternative measure of atherosclerosis. The most commonly used mouse models to study atherogenesis are the ApoE^{-/-} mice and the LDL receptor deficient (LDLR^{-/-}) mice, both characterized by hypercholesterolemia. The normal concentration of serum cholesterol in mice ranges from 80 to 100 mg/dL. ApoE^{-/-} mice and LDLR^{-/-} mice fed a chow diet, on the other hand, have an elevated cholesterol level of 400-500 mg/dL and 175-225 mg/dL respectively [72]. ApoE^{-/-} mice spontaneously develop fatty streaks in a proximal aorta at around 3 months old and aortic plaques at about 8 months old. They tend to have severe atherosclerosis throughout the arterial tree, especially along the aortic arch due to mechanic stress.

LDLR^{-/-} mice, in comparison, barely have any spontaneously developed plaques or lesions on the aorta. When fed an atherogenic diet (i.e., high fat diet), ApoE^{-/-} mice will have abundant plaques and lesions that develop proportionally to the cholesterol intake, while LDLR^{-/-} mice just show medium plaques and fatty streaks. Hence it can be seen that ApoE^{-/-} mice are even more susceptible to hyperlipidemia and atherosclerosis than LDLR^{-/-} mice [73]. The tendency of ApoE^{-/-} mice to develop atherosclerosis has been attributed mainly to the raised lipid level in blood due to loss of ApoE and thus compromised clearance of chylomicrons and VLDL remnants. However, in recent years, emerging studies have begun to uncover

new functions of ApoE that contribute to protection against atherosclerosis, independent of lipid. Although in ApoE^{-/-} mice the lowering of cholesterol level successfully reduced the severity of atherosclerosis and atherosclerotic calcification, it is unknown whether ApoE affects other types of calcifications, such as CKD-induced calcification, which is not directly associated with lipid. Furthermore, among the identified receptors of ApoE, LRP and LDL receptors prefer lipid-bound ApoE as their ligand[74]. On the contrary, the VLDL receptor does not require association of ApoE with lipid for optimal recognition [75]. This indicates lipid-free ApoE might be sufficient to regulate specific cellular functions.

Culture of macrophages with ApoE upregulated ATP binding cassette transporter A 1(ABCA1) expression and increased cholesterol efflux via VLDL receptor and ApoE receptor 2 (ApoER2)[76]. Lipid-free ApoE has antimitogenic effects on VSMCs, by stimulating Cox-2 gene expression [77]. Moreover, atherosclerosis is widely known as a chronic inflammatory disease. Inflammation accompanies atherosclerosis when it goes through different phases. At the early stage of atheroma development, LDL retained at the intima of blood vessels becomes oxidized and these oxidized LDL can stimulate the expression of proinflammatory factors and adhesion molecules like cell adhesion molecule-1 (VCAM-1)[78]. VCAM-1 enables attachment of leukocytes to the vessel walls, which would be impossible during normal conditions. Macrophages are activated in early atherosclerotic lesions and produce TGF- β or reactive oxygen species (ROS) which contribute to calcification [79]. As mentioned above, Runx2, a key regulator of

calcification, colocalizes with calcified areas and is expressed by macrophages in plaque. One secreted cytokine called macrophage colony-stimulating factor (M-CSF) can be found in plaque as well [80]. M-CSF is not only critical for proliferation and differentiation of monocytes and macrophages, but also important for osteoblast survival and function. Therefore, calcification in atherosclerosis represents a combination of osteogenesis and chronic inflammation. Previous data implicates the role of ApoE in inflammation suppression by showing that ApoE inhibits the conversion of macrophages to an antiinflammatory phenotype. In addition, ApoE inhibits lymphocyte proliferation and production of interleukin-2 (IL-2) [81]. Hence, to clarify the effect of ApoE on calcification in an environment without intervention of inflammation or cholesterol, an *in vitro* calcification system is needed, and Pi-treated VSMCs will serve as a suitable cellular model to study the calcification process in the absence of inflammation or lipids. Besides, most of the previous studies compared the atherosclerotic plaques and vascular calcification in relatively old mice (30-60 weeks old), when plaques already have formed in the aorta. To date, no firm evidence has been provided regarding whether mice deprived of ApoE are more likely to develop vascular calcification before plaques are formed.

2. ApoE regulation of canonical Wnt/ β -catenin pathway

The canonical Wnt/ β -catenin pathway depends on Frizzled and LRP5/6 coreceptors for activation. Both LRP5 and LRP6 receptors are required for canonical Wnt/ β -catenin signalling to be generated [82]. Interestingly, ApoE is a ligand for

LRP5 [83] and predicted to bind to LRP6[84] as well. Although ApoE recognition of LRP5/6 as a heterodimer coreceptor remains unclear, the regulatory effects of ApoE on Wnt pathway have been observed.

In colon adenocarcinoma cells, ApoE treatment caused β -catenin to localize mainly in the areas of cell-cell interaction along the cellular membranes, while untreated cells showed a more diffused pattern of β -catenin distribution[85]. ApoE4, one of the three isoforms of ApoE, greatly inhibited canonical Wnt signalling in PC12 cells, possibly by binding to LRP1 and thus suppressing expressions of various Wnt ligands. The other two isoforms of ApoE, ApoE2 and ApoE3 also negatively regulate Wnt/ β -catenin pathway in the presence of LRP5 [86]. Accordingly, it is likely that ApoE acts as a potential mediator of β -catenin pathway. However, whether ApoE regulates vascular calcification via canonical Wnt/ β -catenin signaling remains unknown. By using aortic VSMCs isolated from ApoE^{-/-} mice, we will be able to observe what effects ApoE has on the β -catenin pathway.

3. Regulatory impact of ApoE on GTAP

Although studies have indicated the association of GTAP with several cancers as well as with reproductive capability [87-89], how GTAP affects the cardiovascular system remains elusive. However, based on the yeast two-hybrid system database and membrane based human proteome array, an E3 ligase called STUB1 (CHIP) is predicted to interact with GTAP[90]. Li *et al* have shown that STUB1 directly recognizes and binds to Runx2, a master regulator in osteogenesis. In osteoblasts,

STUB1 targets Runx2 for proteasome-mediated degradation, thus inhibiting osteogenic differentiation [91]. Taken together, it is likely that ApoE mediates the progression of calcification via regulation of GTAP. Our previous data showed lower expression of GTAP in ApoE^{-/-} mice aortas compared to the background strain. Furthermore, ApoE induced the expression of GTAP in a time dependent manner. Whether ApoE regulates calcification via GTAP, however, remains unknown.

Hypothesis: *Endogenous ApoE expression serves as a critical factor that prevents vascular cell osteogenesis and calcification during the pathogenesis of atherosclerosis, by regulating the signal transduction pathways known to be involved in calcification, such as the canonical Wnt/ β -catenin and ubiquitin-proteasome pathways.* Our long-term goal is to define the protective function of ApoE toward vascular cells and the underlying mechanisms of its regulatory roles in calcification.

The **Specific Aims** are:

Aim 1: Examine whether ApoE deficiency accelerates vascular cell calcification in the arterial wall with atherosclerosis.

- a). Observe the progression of calcification and expressions of osteogenic genes in ApoE-deficient aortas by immunohistochemical methods.
- b). Perform *ex vivo* calcification experiments on aortic rings isolated from ApoE^{-/-} mice and assess the extent of calcification using a quantitative calcium assay and Alizarin S staining.

Aim 2: Study whether ApoE^{-/-} VSMCs express high levels of osteogenic genes and develop calcification induced by Pi in culture.

- a). Treat cultured ApoE^{-/-} VSMCs with Pi to determine whether ApoE-deficiency exacerbates calcification *in vitro*.
- b). Perform Western blot and qRT-PCR to detect osteogenic genes in ApoE^{-/-} VSMCs.

Aim 3: Study whether the regulation of ApoE-dependent calcification in VSMCs depends on the canonical Wnt/ β -catenin pathway.

- a). Analyze activation of the Wnt/ β -catenin pathway by quantifying the mRNA levels of β -catenin downstream target genes, as well as observing nuclear translocation of β -catenin in ApoE^{-/-} VSMCs incubated with Pi and ApoE.
- b). Assess whether the effect of ApoE on calcification in ApoE^{-/-} VSMCs is abolished when the Wnt/ β -catenin pathway becomes constitutively activated.

Aim 4: Determine how GTAP regulates calcification and whether the mediation of ApoE-dependent calcification partially depends on GTAP.

- a) Analyze the level of GTAP during calcification and the severity of calcification as well as levels of calcification-related genes when expression of GTAP alters.
- b) Determine whether GTAP contributes to the impact of ApoE on calcification by examining the extent of calcification in ApoE^{-/-} VSMCs.
- c) Assess whether GTAP affects stability of osteogenic genes by increasing ubiquitylation.

Chapter 3 : Methods and Materials

1. Animals

ApoE^{-/-}, GTAP^{-/-}, C57BL/6J and 129sv (used as wild type control animals) mice were housed and bred under specific pathogen-free conditions in the Animal Care Facility at the University of Texas Health Science Center and all animal study protocols were approved by the Animal Welfare Committee of the University of Texas Health Science Center. The animal experiments were performed following the guidance of NIH and university regulations.

1.1 *Ex vivo* calcification assays in mouse aortic rings exposed to phosphate

Male mice of 8-10 weeks old were used. Individual mice were put in sealed chambers and anesthetized by isoflurane inhalation for 30-60 seconds. We ensured the mice were anesthetized by lightly pinching the rear foot pad. If a knee jerk was elicited, the mice were put back into the chamber. After the mouse was anesthetized, cervical dislocation was performed. Then its skin was disinfected with 70% ethanol and chest was cut open. Aortas were dissected out from aortic arch to the abdominal aorta and perivascular fat was removed. Aortas were cut into 2-3 mm length aortic rings that were rinsed with sterile PBS (HyClone, Logan, UT, Cat# SH3025601) containing 200 U/mL penicillin and 200 µg/mL streptomycin for at least 5 times. Aortic rings were then cultured in individual wells of 24-well plates in DMEM (Sigma-Aldrich, St Louis, MO, Cat#D5796) containing 100 U/mL penicillin, 100

$\mu\text{g/mL}$ streptomycin, 5% FBS and 1mmol/L phosphate. Calcification was induced by culture in high phosphate (2.6mM) media for 9 days. Medium was changed every 3 days. At the termination of the experiment, aortic rings were decalcified with 0.6 M HCl at 37°C for 24 hours. After decalcification, aortic rings were dried at 37°C and weighed. The calcium content of the supernatant was determined colorimetrically with the o-cresolphthalein complexone kit from Abcam (Cambridge, UK, Cat#102505), standardized to the dry weight of aortic rings.

1.2 Histopathology and immunofluorescence of aorta sections

Aortas were harvested from male mice of 50-55 weeks old for immunofluorescence studies. Freshly dissected aortas were placed onto a pre-labeled tissue base mold. The entire tissue block was later covered with OCT (Tissue-Tek; Sakura Finetek USA, Cat#4583) and left on dry ice for 15-20 minutes until it was completely frozen. Frozen tissue blocks were stored at -80°C until ready for sectioning. Prior to sectioning, the frozen tissue block was transferred to a cryotome cryostat (Leica, Buffalo Grove, IL). The tissue block was sectioned to a 5 μm thickness using the cryotome. Sections were placed onto glass slides suitable for immunohistochemistry. Sections can be stored in a sealed slide box at -80°C for later use.

For immunohistochemical analysis, sections were dried at 37 °C for 15 minutes and fixed in pre-cooled acetone (-20°C) for fixation for 10 minutes. After fixation, slides were left at room temperature for 20 minutes or more to allow residual

acetone to evaporate. Slides were rinsed with PBS 3 times, 5 minutes each. Specimens were blocked in blocking buffer (5% FBS and 5% BSA in PBS with 0.1% Tween-20) at room temperature for 60 minutes. Primary antibodies including goat anti-SM22 α 1:200 (Abcam, Cat#10135), rabbit anti- α SMA 1:200 (Abcam, Cat#5694), rabbit anti-BMP2 1:200 (Abcam, Cat#14933) and goat anti COLI 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA, Cat#59772) were prepared in blocking buffer. Sections were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies TRITC goat anti-rabbit IgG (H+L) conjugate 1:1000 (Invitrogen, Waltham, MA, Cat#81-6114) and TRITC rabbit anti-goat IgG(H+L) 1:1000 (Invitrogen, Waltham, MA, Cat#31650) at room temperature for 60 minutes. Sections were mounted with mounting medium containing DAPI (Vectashield, Burlingame, CA, Cat#H-1500). Fluorescence was observed and photographed with a fluorescent microscope (Nikon Eclipse TE2000-U).

1.3 Alizarin Red S staining of aorta sections

Aortas were harvested from male mice of 50-55 weeks old for Alizarin Red S staining to detect calcium deposition. Frozen sections of aortas were cut at 4 μ m, dried and fixed in acetone as mentioned above; 2% Alizarin Red S staining solution was prepared freshly by dissolving Alizarin Red S powder in PBS. The pH of Alizarin Red S staining solution was adjusted to 4.1-4.3 by adding 0.5% ammonium hydroxide and filtered to remove undissolved powder. Sections were stained with Alizarin Red S

solution for 10 minutes and washed in 70% ethanol for 15 minutes. Sections were then observed under the microscope. Calcium deposits appear as orange-red colour after staining.

2. Cell Culture

2.1 Culture of VSMCs

Aortas were harvested from 5-6 week old mice sterilely. The whole aorta was rinsed with ice-cold PBS 3 times, and then transferred to warmed DMEM supplemented with 20% FBS and washed with new medium for 3 times. The aorta was cut open longitudinally, and the endothelium was gently scrapped off with finely toothed forceps or a scalpel and minced with fine micro-dissecting scissors into approximately 1 mm² squares. Following dissection procedures, the tissue pieces were digested in a conical shaker flask (Costar) with constantly stirring at 37 °C for 8 minutes. After trypsinization, endothelial cells and fibroblasts were dispersed from the tissue. The tissue pieces were then subjected to a second digestion. Resulting suspensions were discarded from each digestion procedure. The digestion of the tunica was terminated by adding DMEM medium supplemented with 20% FBS. The undigested tissue pieces were evenly distributed into a T25 tissue culture flask (about 15 pieces per aorta, with a distance of 1 cm between individual pieces, no medium added into the flask), which was put upright at 37 °C into a CO₂ incubator with a humidified atmosphere (5% CO₂, 95% air, Thermo, USA) in order to facilitate the attachment of pieces on the surface of the flask; to remove fibroblasts from the culture,

the medium was aspirated from the flask and the cells were washed with 25 mL PBS (per flask). The PBS was aspirated and 7 mL of cold Trypsin-EDTA (0.25%) was added to the flask. After incubating for 2 min at room temperature the flask was rapped with the open palm to release the fibroblasts and the Trypsin-EDTA was aspirated. The cells were washed with 25 mL PBS, the wash aspirated, and 35 mL of fresh medium was added. The culture was then viewed under the microscope to determine if the fibroblasts were removed. The new cell culture was then incubated at 37 °C with 5% CO₂. The treatment was repeated if fibroblasts proliferated again in the culture. 4–6 h after inoculation, the flask was put horizontally and then 2 mL of DMEM containing 20% FBS was carefully added to the flask. The culture medium was replenished after 72 h and thereafter every 48 h.

Cells were passaged when they reached 80-90% confluence. Prior to splitting cells, cell culture medium(DMEM containing 10% FBS), PBS and trypsin-EDTA solution to 37 °C by placing all solutions in water bath set at 37 °C. All culture medium was removed from the cell culture flask carefully. Cells were washed with PBS and trypsin-EDTA was added into the flask. The flask was incubated in the cell culture incubator (37 °C) for 2-3 minutes. Cell morphology was observed under the microscope. When cells started appearing rounded, the flask was gently tapped to remove the cells completely from the surface. Cells were washed out from the surface by pipetting the fresh complete culture medium (5 ml) all over the surface. Certain volume of the cell suspension, depending on the split ratio, was taken out and added into a new culture flask. Fresh complete medium was added into the new flask, which

was put into the 37 °C incubator. The remainder of the cells can be discarded, used for an experiment or for making more culture flasks.

2.2 Treatment of VSMCs

VSMCs from passage 5-10 were used for all the *in vitro* experiments. Calcification medium was made by adding NaH_2PO_4 (pH adjusted to 7.4) into 5% FBS DMEM medium to obtain a final concentration of 3.6mM inorganic phosphate (Pi). VSMCs cultured in 5% FBS DMEM (0.9mM phosphate) were used as controls. To determine β -catenin activation, cells were treated for 1 day. Under other circumstances, culture medium was changed every 2-3 days for up to 9 days; 6 $\mu\text{g}/\text{mL}$ ApoE(Athens Research and Technology, GA, Cat#16-16-120500) and/or 20ng/mL Wnt3a(R&D Systems, Minneapolis, MN, Cat#1324-WN-002) was added into medium as needed. To study the effect of ApoE on GTAP expression in VSMCs, cells were starved with DMEM without serum for 24 hours before treatment. To determine the stability of Runx2 protein, VSMCs were treated with 80 $\mu\text{g}/\text{mL}$ cycloheximide (Sigma-Aldrich, Cat#C1988) for up to 4 hours in regular culture DMEM medium.

2.3 Detection of calcification in cell culture

For detection of calcification in VSMCs, the calcium assay or Alizarin Red S staining was performed. Procedures are similar to those for aortic sections with minor modifications. For Alizarin Red S staining, cultured medium was aspirated and cells

were washed with PBS 3 times; 2.5% glutaraldehyde was used to fix cells at room temperature for 15 minutes. Then cells were rinsed with PBS (pH adjusted to 4.1-4.3) 3 times and incubated with 2% Alizarin Red S solution at 37 °C for 20 minutes. Excess dye was washed off cells with PBS and staining was observed under the microscope. For calcium assay, cultured medium was aspirated and cells were washed with PBS 3 times. Then 0.6M HCl was used to incubate VSMCs at 37 °C overnight to dissolve calcium deposition. The supernatant was taken and the calcium content was quantified using a colorimetric calcium assay kit as mentioned above. Output was measured at 575nm and calcium content was calculated using a formula derived from the standard curve. Cells were washed with PBS, lysed with 0.1 mol/L NaOH/0.1% SDS. Protein content was determined with the BCA protein assay kit (Thermo Scientific, Waltham, MA, Cat#23225) and calcium content was normalized to total protein content.

2.4 Immunofluorescence in VSMCs

VSMCs were seeded and cultured on cover slides in 24-well plates. At the endpoint of treatment of cells, culture medium was aspirated and cells were washed with PBS twice. Cells were fixed in 4% paraformaldehyde (PFA) solution diluted in warm PBS at room temperature for 10 minutes, then rinsed again with PBS 3 times at room temperature, 5 minutes each; 0.3% Triton X-100 in PBS was applied to permeabilize cells for 10 minutes with shaking, followed by washing with PBS for 3

times, 5 minutes each. Cells were incubated with blocking solution (5% FBS and 5% BSA in PBS with 0.1% Tween-20) at room temperature for 60 minutes. Primary antibody rabbit anti- β -catenin 1:200(Santa Cruz Biotechnology, Santa Cruz, CA, and Cat #7963) diluted in blocking solution was used to incubate cells at 4 °C overnight.

Cells were washed with PBS 3 times, 5 minutes each and incubated with secondary antibody TRITC goat anti-rabbit IgG (H+L) conjugate 1:1000(Invitrogen, Waltham, MA, Cat#81-6114) diluted in blocking solution at room temperature for 60 minutes. After cells were washed with PBS at room temperature 3 times, 5 minutes each, DAPI solution (Sigma-Aldrich, St Louis, MO, Cat#62248) was applied to counterstain cells, which were observed under the microscope.

3. ApoE cDNA Subcloning and Transfection

Plasmid Sport6 that contains mouse ApoE cDNA was constructed by senior research scientist Michael Wassler and used as the template for ApoE cDNA subcloning. Primers applied for amplifying ApoE cDNA are: 5'-CACCCAATCACAATTGCG-3' (sense) and 5'-TTGATCTCCTGGGCCAC-3' (antisense). The PCR reaction conditions are as follows: denaturing at 94 °C for 15 seconds, annealing at 52 °C for 30 seconds, extending at 68 °C for 1 minute. In total, 35 cycles were performed.

After PCR, the reaction mix was separated on 0.8% agrose gel by electrophoresis. A band of the predicted size was excised under the observation of UV visualization. The DNA in the cut gel was isolated by using QIAquick Gel Extraction Kit (Invitrogen, Cat#28704). First, three volumes of buffer QG was added to one

volume gel and incubated at 50 °C for 10 minutes until the gel was completely dissolved. Then one volume of isopropanol was added and the sample was transferred to a QIAquick column for spinning. Buffer PE was used for washing the spin tube pellet, followed by DNA elution with buffer EB.

Isolated DNA was ligated into pcDNA3.1D/V5-His-TOPO cloning plasmid (Invitrogen, Cat#K900001). The ligation reaction system contained 2 µL fresh PCR product, 1 µL salt solution, 1 µL pcDNA TOPO vector, and 2 µL sterile water. The reaction was mixed gently and incubated at room temperature (22-23 °C) for 5 minutes. For transformation, 2 µL reaction mixture was added into one vial of One Shot TOP10 chemically competent *E. coli* (Invitrogen, Cat#C4040-10) and mixed gently. The *E. coli* was then incubated on ice for 30 minutes, followed by heat shock at 42 °C for 30 seconds without shaking. Immediately after heat shock, tubes were transferred to ice and 250 µL S.O.C medium was added into tubes. Tubes were capped tightly and shaken horizontally (200 rpm) at 37 °C for one hour; 100-200 µL from each transformation was spread on a prewarmed selective plate and incubated at 37 °C overnight.

Five or more colonies were picked and cultured in 5 mL LB medium containing 100 µg/mL ampicillin. Plasmids were isolated using QIAprep Spin Miniprep Kit (QIAGEN, Venlo, Netherlands, Cat#27104). Bacterial cells were suspended in 250 µL P1 buffer and transferred to a microcentrifuge tube; 250 µL P2 buffer was then added and tubes were inverted gently 4-6 times, after which 350 µL N3 buffer was added. Tubes were immediately inverted gently 4-6 times. Samples

were centrifuged at 13,000rpm for 10 minutes in a table-top microcentrifuge. The supernatants were applied to a QIAprep spin column by pipetting. Columns were centrifuged for 1 minute; flow-through was discarded, and then washed with 0.75mL PE buffer for 1 minute by centrifuging. Samples were centrifuged again for 1 minute and the column was put in a clean microcentrifuge tube; 25 μ L EB buffer was added to the column to elute DNA. The concentration of DNA isolated was measured in a spectrophotometer (Beckman Coulter). Samples were sent to SeqWright Laboratory (Houston,TX) for sequencing to confirm that the ApoE gene is cloned and in frame.

The pcDNA3.1D/V5-His-TOPO plasmid that contains the ApoE gene was designated as ApoE cDNA and the empty plasmid was referred to as the vector. For transfection, 1.2 μ g DNA dissolved in EB buffer was diluted with medium without serum, protein or antibiotics to a total volume of 100 μ L. Then 4.5 μ L Attractene Transfection Reagent (Qiagen, Cat#1051561) was added and mixed by pipetting up and down. The mixed liquid was incubated at room temperature (15-25 $^{\circ}$ C) for 15 minutes to allow transfection complex formation. The culture medium was aspirated and 1mL fresh medium was added into each well of 6-well plates. Transfection complexes were added drop-wise onto the ApoE^{-/-} VSMCs. Plates were swirled gently to ensure uniform distribution of the transfection complexes. 72 hours after transfection, VSMCs were passaged into selective medium that contains 800 μ g/mL neomycin and the medium was changed every 3 days. When VSMCs reached 90% confluence, cells were passaged again and experiments were performed.

4. Production of Lentivirus Vector for GTAP Overexpression

4.1 GTAP cDNA subcloning

Plasmid pET100D that contains murine GTAP cDNA was constructed by senior research scientist Dr Michael Wassler and used as the template for GTAP cDNA subcloning. Primers applied for PCR reaction are: 5'-GGAATTCGAGCGGAGCGGAGGATGCAG-3' (sense) and 5'-CGGGATCCGCCATCTTCCTTTGGGGGTGTGTA-3' (antisense). PCR product of predicted size was isolated and later digested by restriction endonuclease EcoRI and BamHI (New England Biolabs, Ipswich, MA) in 37 °C water bath for 1 hour. Digested PCR product was ligated with p3XFLAG-CMV™-14 Expression Vector (Sigma-Aldrich, Cat#E7908), which has also been digested by EcoRI and BamHI. Ligation was performed with DNA Ligation Kit (Takara, Japan, Cat# 6023).

The digested plasmid and PCR product were combined to a total volume of 10µL. One volume of Ligation Mix from the Ligation Kit was added to the DNA solution and mixed thoroughly. The mixed solution was incubated at 16 °C overnight, then directly used for transformation with One Shot TOP10 chemically competent *E.Coli* cells. After transformation, *E.Coli* cells were cultured on ampicillin selective plates overnight at 37 °C. Colonies were picked and amplified in LB, after which plasmid DNA was isolated and sequenced to confirm the GTAP gene was successfully inserted into the p3XFLAG-CMV™-14 vector.

To mutate the active cysteine residue in the C-terminus of GTAP into an

alanine, the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, Cat#200521) was used. Primers were designed using the online primer design tool on the Agilent Technologies website.

After constructs were sequenced, wildtype GTAP gene or mutated GTAP (C351A) with 3XFLAG tag was cloned into CD513B-1 lentivirus expression vector. Plasmids were isolated from bacterial cells using Plasmid MaxiPrepKit (Qiagen, Cat#12162).

4.2 Packaging and production of lentiviral particles

293ft cells (ATCC, Manassas, VA, Cat#R700-07) were plated onto 150cm² plates and cultured in DMEM supplemented with 10% FBS. When cells reach 60-80% confluence, 4.5µg CD513B-1 empty plasmid or CD513B-1 plasmid that contains either wildtype GTAP or mutated GTAP was mixed with 45µL pPACKH1 packaging plasmids (System Biosciences, Los Gatos, CA, Cat#LV500A-1) in DMEM. Lipofectamine LTX and Plus Reagent (Life Technologies, Cat#15338100) was used to transfect 293ft cells. After transfection, plates were returned to the cell culture incubator at 37 °C with 5%CO₂.

5. Production of Lentivirus for GTAP Knockdown

5.1 Design and Cloning of GTAP shRNA

GTAP shRNA DNA templates (9 in total) were designed using online design tools siDirect and Darmocon and Ambion, then synthesized by Sigma-Aldrich. The

following primers were used to amplify desired GTAP shRNA sequence:
5'-GAGCTGGAGCCATGCGAATTC -3' (sense) and 5'-
GACGAGTCGACGAGGTACCGG-3' (antisense). PCR products of predicted size
were enzymatically digested by restriction endonucleases EcoRI and AgeI, followed
by ligation with pLKO.1 shRNA cloning vector (from Dr Jianping Jin's lab). Colonies
grown on selection plates which contain ampicillin were picked and sequenced to
confirm the GTAP shRNA sequences are correct.

5.2 Packaging and production of lentiviral particles

Lentiviral particles were produced following similar procedures mentioned
above. 293T cells (ATCC, Manassas, VA, Cat#R700-07) were plated onto
150cm² plates and cultured in DMEM supplemented with 10% FBS. When cells
reached 60-80% confluence, 4.5µg pLKO.1 empty plasmid or pLKO.1 plasmid that
contains GTAP shRNA was mixed with packaging plasmids (from Dr Jianping Jin's
lab) in DMEM. Lipofectamine LTX and Plus Reagent (Life Technologies,
Cat#15338100) was used to transfect 293T cells. After transfection, plates were
returned to the cell culture incubator at 37 °C with 5%CO₂.

6. Western Blot Analysis

6.1 Sample preparation

VSMCs were first washed with PBS 3 times before Trypsin-EDTA (ATCC,
Cat#30-2101) was added onto culture plates. Plates were incubated in the 37°C

incubator for 10-20 minutes until cells detached from the bottom of plates. Cells were centrifuged at 1000 RPM and were resuspended with PBS. Resuspended cells were centrifuged again to obtain cell pellets. PBS was aspirated and ice-cold RIPA Lysis and Extraction Buffer (Life Technologies, Cat#89900) with Protease Inhibitor Cocktails (Sigma-Aldrich, Cat#p8340) and Phosphatase Inhibitor Cocktails (Sigma-Aldrich, Cat#5726 and Cat#0044) was mixed with pellets in eppendorf tubes. Tubes were placed on ice for 15 minutes and centrifuged at 15,000 RPM for 10 minutes. Supernatant was collected and protein concentration of each sample was measured using BCA protein assay kit (Thermo Scientific). The desired amount of protein sample was mixed with protein loading buffer and boiled at 98°C for 7 minutes before Western blot was performed.

6.2 Loading and running SDS-PAGE gel

Equal amounts of protein were loaded into each well of all SDS-PAGE gel, along with Precision Plus Protein Standards molecular weight marker (Bio-Rad, Cat# 161-0374). A constant voltage of 100 V was used to run the gel for 1-2 hours.

6.3 Electrotransferring of proteins from SDS-PAGE gels to membranes

PVDF membranes were activated with methanol for 1 minute, then drained and equilibrated for 5 minutes in the desired transfer buffer. The filter paper and fiber pads were soaked in transfer buffer also and the blot 'sandwich' was assembled

according to the instructions provided by Bio-Rad. A constant current of 300mA for 2 hours was used to complete the transfer process. After the transfer, the blot 'sandwich' was unclamped and the sheets of filter papers were removed to expose the gel. The membrane was then taken out and marked properly.

6.4 Immunostaining of target protein bands using antibodies

The membrane was rinsed with PBS for 10 minutes, and then blocked with 10% BSA in TBS-T for at room temperature for 1 hour. After blocking, the membrane was incubated with primary antibodies diluted in 3% BSA in TBS-T at 4 °C overnight. Primary antibodies used include goat anti-SM22 α 1:5000 (Abcam, Cat#10135), rabbit anti- α SMA 1:1600 (Abcam, Cat#5694), rabbit anti-Runx2 1:1000 (Cell Signaling, Cat#8486), rabbit anti-GTAP 1:1000 (provided by Dr Michael Wassler), mouse anti- β -catenin 1:1600 (Santa Cruz Biotechnology, Cat#133240), goat anti-lamin B1 1:800 (Santa Cruz Biotechnology, Cat#6216), mouse anti-FLAG 1:1000 (Sigma-Aldrich, Cat# F3165), rabbit anti-GAPDH 1:30,000 (Abcam, Cat#8245) and mouse anti-ApoE 1:2000 (Abcam, Cat#1906). The membrane was washed with TBS-T 3 times, 10 minutes each and incubated with secondary antibodies diluted in 3% BSA in TBS-T at room temperature for 1 hour. Secondary antibodies used include goat anti-rabbit IgG HRP 1:20,000 (Santa Cruz Biotechnology, Cat# 2030), goat anti-mouse IgG HRP 1:5,000 (Santa Cruz Biotechnology, Cat#2005) and donkey anti-goat IgG HRP 1:5000 (Santa Cruz Biotechnology, Cat#2020). The membrane

was rinsed with TBS-T 3 times, 5 minutes each. Western blotting ECL substrate (Pierce, Cat#32106) was used for developing. The membrane was incubated in ECL working solution for 1 minute at room temperature, after which it was placed in a plastic sheet protector. An absorbent tissue was used to remove excess liquid and to carefully press out bubbles from between the plastic protector and the membrane. The protected membrane was put in a film cassette with the protein side facing up. Exposure was done using either X-ray films or developing machine (Bio-Rad, Universal Hood II).

7. Quantitative Reverse Transcription and Polymerase Chain Reaction

7.1 RNA isolation

Culture medium was removed and TRIzol Reagent (Life Technologies, Cat#15596) was added onto VSMCs. Cells were lysed by pipetting up and down until no cell pellets are seen and transferred to an eppendorf tube. Then the homogenized sample was incubated at room temperature for 5 minutes to permit complete disassociation of the nuclear protein complex; 0.2mL chloroform was added per 1mL TRIzol Reagent for homogenization. Tubes were capped securely and shaken vigorously by vortex machine for 15 seconds, after which the sample was incubated at room temperature for 2-3 minutes. The sample was centrifuged at 12,000 x g for 15 minutes at 4°C.

After centrifugation, the mixture separates into a low red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase was

removed by angling the tube at 45° and pipetting the solution out. The aqueous phase was put in a new tube and 0.5mL ice-cold 100% isopropanol per 1mL of TRIzol Reagent used for homogenization was added and mixed with the aqueous phase. The mixture was incubated at room temperature for 10 minutes and then centrifuged at 12,000 x *g* for 10 minutes at 4°C. The supernatant was removed and the RNA pellet was left in the tube. The pellet was washed with 75% ethanol. The sample was vortexed briefly and centrifuged at 7,500x *g* for 5 minutes. The wash was discarded and the RNA pellet was air-dried for 5-10 minutes. The RNA pellet was resuspended in RNase-free water by passing the solution up and down through the pipette tip several times. The sample was incubated in a heat block at 55-60 °C for 10 minutes and stored at -80°C until use.

7.2 Reverse transcription

For first-strand cDNA synthesis, SuperScript® III First-Strand Synthesis SuperMix (Invitrogen, Cat#18080-400) was used to convert RNA into first-strand cDNA. Each component was briefly centrifuged. Up to 5µg total RNA was combined with oligo(dT) primer, annealing buffer and RNase/DNase-free water in a thin-walled PCR tube on ice. The mixed solution was incubated in a thermal cycler preheated to 65°C for 5 minutes, then immediately placed on ice for at least 1 minute. The contents of the tube were collected by brief centrifugation. 2X First-Strand Reaction Mix and SuperScriptR III/RNaseOUT™ Enzyme Mix were added into the tube on ice to a total

volume of 20 μ L. The sample was vortexed briefly to mix by brief centrifugation, and then incubated at 50°C for 50 minutes. The reactions were terminated at 85°C for 5 minutes and chilled on ice. The reaction was either stored at -20°C or used for PCR directly.

7.3 Quantitative polymerase chain reaction

To assess gene expression with real-time quantitative PCR, iQ™ SYBR® Green Supermix (Bio-Rad, Cat#1708880) was mixed with primers, cDNA template and nuclease-free water in the reaction tube. The reaction mix was centrifuged briefly in a microcentrifuge. Reaction replicates were loaded into PCR tubes and the reaction vessels were sealed. For best results, reaction vessels were briefly spun at low speed to remove any bubbles. The iCycler iQ™ thermocycler (Bio-Rad) was programmed according to recommended real-time PCR protocol with a melt curve step. The sealed reaction vessels were placed in the thermal cycler block, and running was started using a specific PCR protocol. The expression levels of the target mRNAs were normalized to GAPDH mRNA. The primers for PCR amplification are indicated in Table 1.

Gene	Sense Primer	Antisense Primer
Type I Collagen	5'-TAGGCCATTGTGTATGC AGC-3'	5'-ACATG TTCAGCTTTGTG GACC-3'
Alkaline Phosphatase	5'-CACAATATCAAGGATAT CGACGTGA-3'	5'-ACATCAGTTCTGTTCTT CGGGTACA-3'

Runx2	5'-TTACCTACACCCCGCCA GTC-3'	5'-TGCTGGTCTGGAAGGGT CC-3'
Osteopontin	5'-TGGCTATAGGATCTGGG TGC-3'	5'-ATTTGCTTTTGCCTGTTT GG-3'
Osteocalcin	5'-GGGCAATAAGGTAGTGA ACAG-3'	5'-GCAGCACAGGTCCTAAA TAGT-3'
Axin2	5'-CTGGCTCCAGAAGATCA CAA-3'	5'-AATTCGTCACTCGCCTT CTT-3'
CyclinD1	5'-GCCGAGAAGTTGTGCAT CTA-3'	5'-TGAAC TTCACATCTGTG GCA-3'
Tcf4	5'-TTTGCCGTCTTCAGTCT ACG-3'	5'-GCATGAAGAAGGAGCT AGGG-3'
GAPDH	5'-GGTGAAGGTCGGTGTG AACG-3'	5'-CTCGCTCCTGGAAGATG GTG-3'

Table 1. The list and sequence of primers used in quantitative RT-PCR.

8. Reverse transcription and polymerase chain reaction

To confirm ApoE cDNA was successfully inserted into pcDNA plasmid, RT-PCR was performed using the OneStep RT-PCR Kit (Qiagen, Cat#210210). RNA isolated from VSMCs transfected with ApoE cDNA or empty plasmid was used and a master mix solution including RNase-free water, primers, OneStep RT-PCR enzyme mix and 5XOneStep RT-PCR Buffer was made according to the manual. The

sequences of primers used are as following: 5'- AGCTCGGATCCAGTACCCTT-3' (sense) and 5'-CCGCCTCAGAAGCCATAGAG-3' (antisense). The master mix was mixed thoroughly and dispensed appropriately into PCR tubes. Template RNA (1µg) was added to the individual PCR tubes. The PCR reaction conditions are as follows: reverse transcription at 50 °C for 30 minutes, initial PCR activation step at 95 °C for 15 minutes. Then 3-step cycling was set as denaturing at 94 °C for 15 seconds, annealing at 52 °C for 30 seconds, extending at 72 °C for 1 minute. In total, 40 cycles were performed. After PCR, the reaction mix was separated on 2% agarose gel by electrophoresis. Bands of the predicted size were visualized using the imaging machine (Bio-Rad, Universal Hood II).

9. Statistical Analysis

Results are graphed as means \pm standard deviation (SD). Statistics (t test and ANOVA) were performed using GraphPad Prism (GraphPad Software, Inc., CA). A value of $P < 0.05$ was considered statistically significant.

Chapter 4 : Results

1. Increased calcification and pro-osteogenic protein expression in the aortic tissue of ApoE^{-/-} mice

Aortas were dissected from ApoE^{-/-} and WT mice around 50 weeks old on the chow diet. Alizarin S staining, which visualizes calcium deposition in blood vessels, is also greater in ApoE^{-/-} mice while no obvious staining was detected in WT ones (Figure 3a and 3b). Both ApoE^{-/-} and WT mice have positive SM22 α and α SMA aortic fluorescence staining, with WT mice exhibiting a much more intense signal of these smooth muscle lineage markers (Figure 3c-3f). We observed weak signal of calcification marker type I collagen (COL1) in WT mice, while enhanced immunofluorescence was seen in ApoE^{-/-} murine aortas (Figure 3g and 3h). There was almost no detectable BMP-2 staining in WT mice; in ApoE^{-/-} mice, positive signal of BMP-2 fluorescence was found (Figure 3i and 3j). These data indicate that ApoE^{-/-} mice spontaneously develop more aortic vascular calcification than the background strain. *Ex vivo* calcification of aortic rings isolated from ApoE^{-/-} and WT mice was induced by culture with Pi (2.6mM) in medium for 9 days. There was a significant difference between calcium levels of WT and ApoE^{-/-} aortic rings after treatment (ApoE^{-/-} vs. WT: 32.10 \pm 9.68 vs. 11.32 \pm 6.31 μ g/mg, p <0.05) (Figure 3k).

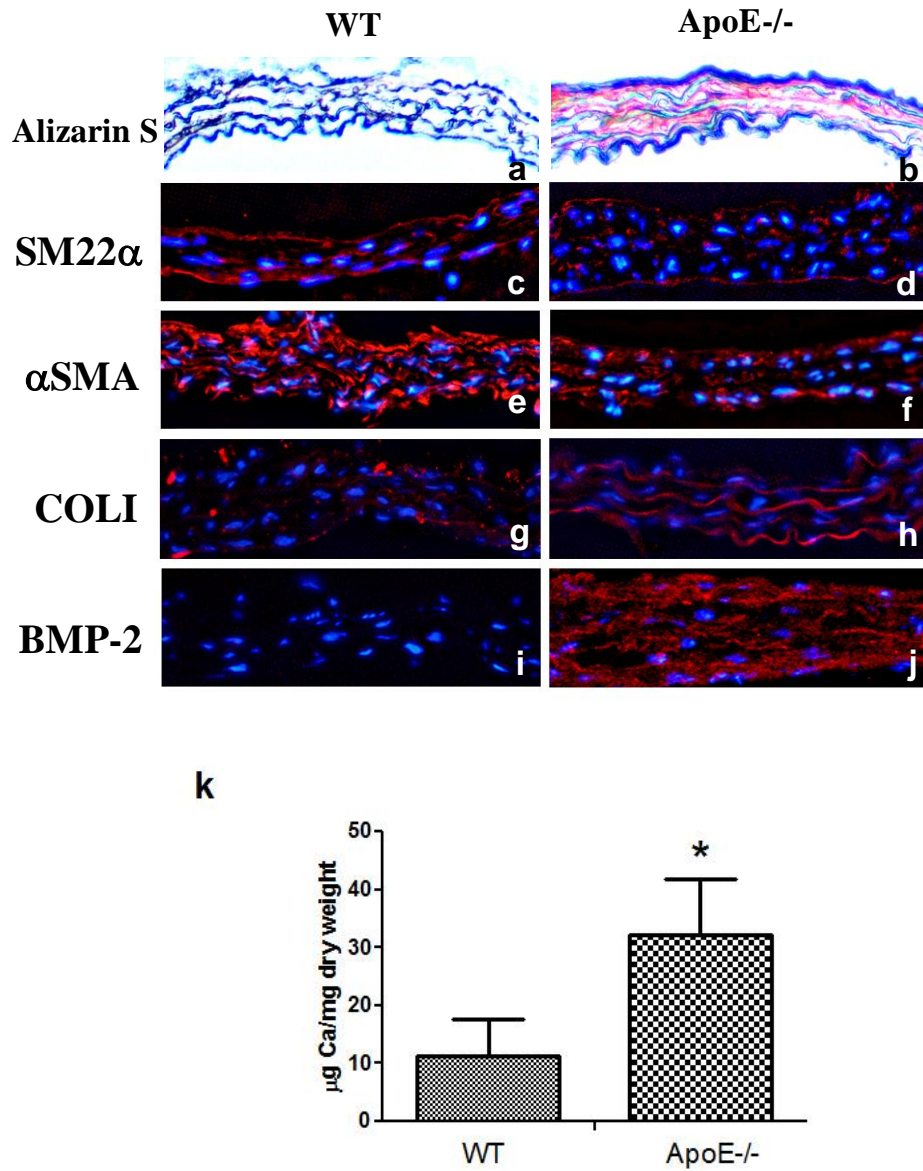


Figure 3. Analysis of calcium contents and biomarkers for smooth muscle and osteogenesis in the aortic tissues of wild type (WT) and ApoE^{-/-} mice. Alizarin S staining was used on aortic sections and positively stained areas are of red color (a and b). Expressions of SM22 α , α SMA, COLI and BMP-2 were detected by immuofluorescence analysis in the aortas, COLI indicates type I collagen (c-j). k: Calcium levels of explanted murine aortic rings after culture in medium containing 2.6mM Pi. n=5 per group. Bars indicate mean \pm S.D. * $p < 0.05$.

2. ApoE^{-/-} VSMCs are prone to Pi-induced calcification in vitro

VSMCs grown in DMEM media with or without Pi (3.6mM) for up to 9 days were stained with Alizarin Red S (2%) for calcium content determination. In cell-free media, there was no detectable calcium precipitation in the wells with VSMC (data not shown), indicating the appearance of calcium deposition in cell culture did not result from chemical reaction of phosphate and components in culture medium. Neither wild type (WT) nor ApoE^{-/-} VSMCs calcified in regular culture medium, but both showed accumulation of calcium deposit when cultured in calcification medium containing 3.6mM phosphate for up to 9 days (Pi). However, ApoE^{-/-} VSMCs exhibited a more drastic increase of calcium mineralization than WT VSMCs in a time dependent manner, manifested by Alzarin S staining (Figure 4a-4h) and calcium assay (Figure 4i). The protein expression level of smooth muscle lineage markers SM22 α and α SMA were decreased in ApoE^{-/-} VSMCs, though no significant difference of Runx2 expression was detected between ApoE^{-/-} and WT cells (Figure 5a-5d). The mRNA level of calcification marker COL1 is upregulated, when calcification suppressor OPN(osteopontin) is downregulated in ApoE^{-/-} cells(Figure 6a and 6b).

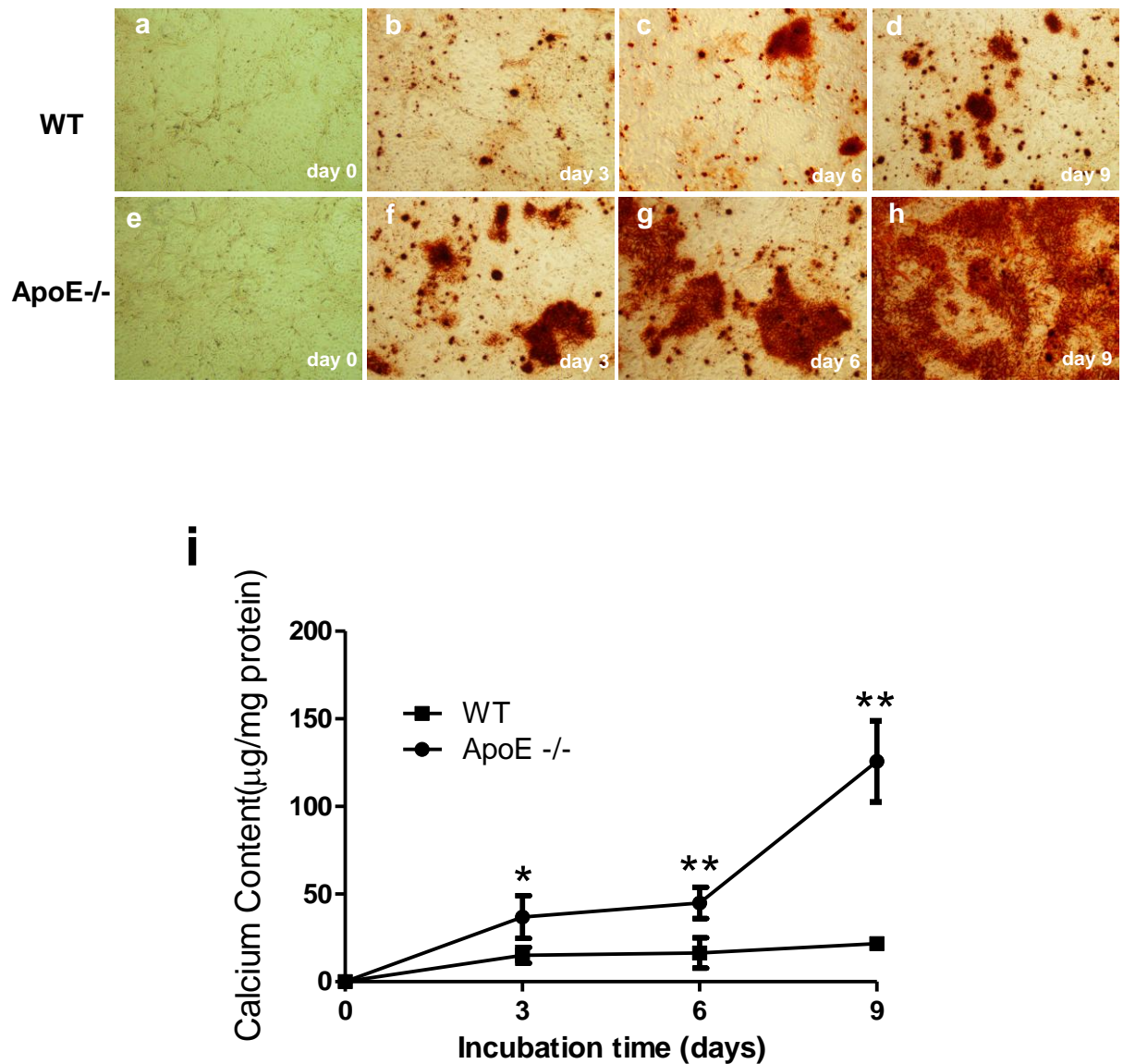


Figure 4 .Analysis of calcium content in WT and ApoE-/- aortic VSMCs incubated with or without Pi. Aortic VSMC from WT and ApoE-/- aortas were incubated in the media containing 3.6mM Pi for up to 9 days, stained with Alizarin Red S for 10 minutes at 37°C, and then visualized under a Nikon microscope. **a-h**, Calcification in WT and ApoE-/- VSMCs incubated with Pi for 0,3,6 and 9 days. Images were taken using a 10x objective.**i**, Colorimetric calcium assays based on the chemical reaction between calcium and o-cresolphthaleincomplexone. n=4,

* $p < 0.05$, ** $p < 0.01$.

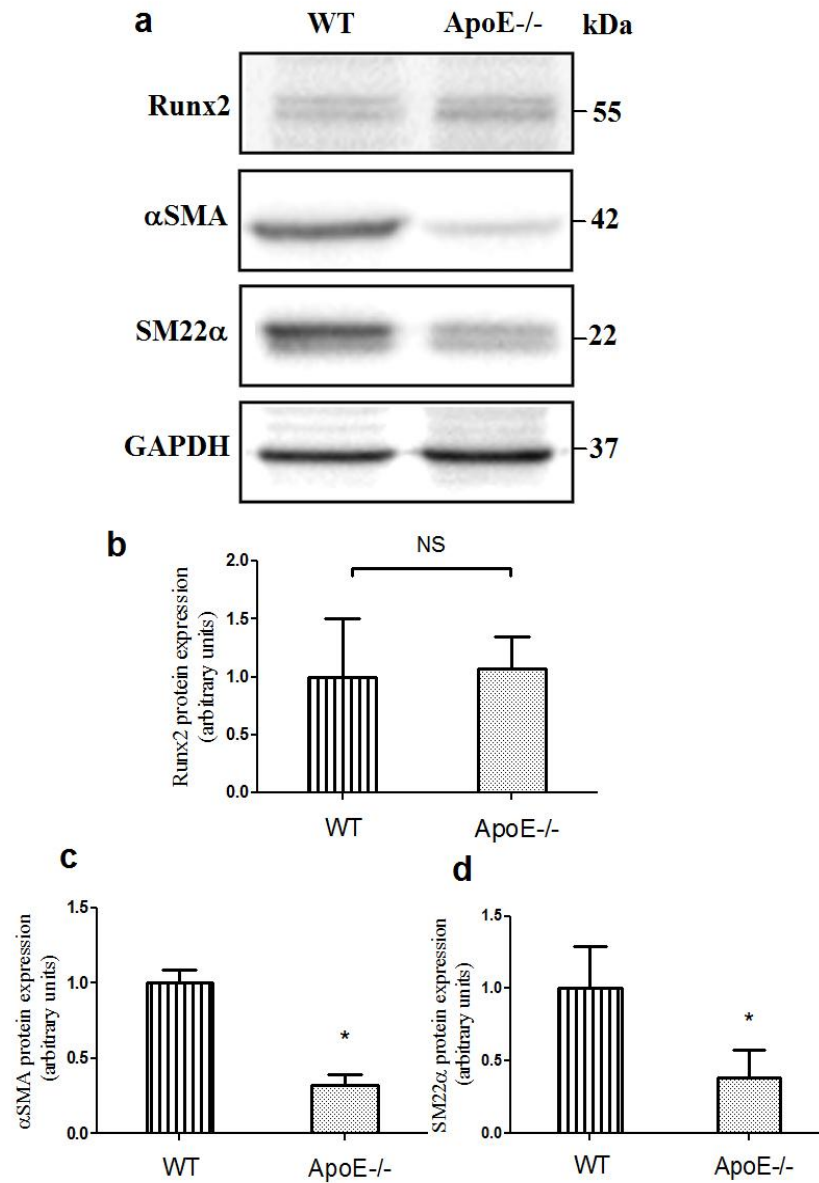


Figure 5. Western blot analysis of smooth muscle-specific and osteogenic biomarkers in wild type and ApoE^{-/-} VSMCs. Protein expressions of SM22α, αSMA and Runx2 are compared between ApoE^{-/-} and WT aortic VSMCs by immunoblotting. A representative western blot is shown at the top (**a**). Bars indicate fold change (n=3) determined by densitometry of target gene bands, normalized to

GAPDH (b-d). * $p < 0.05$.

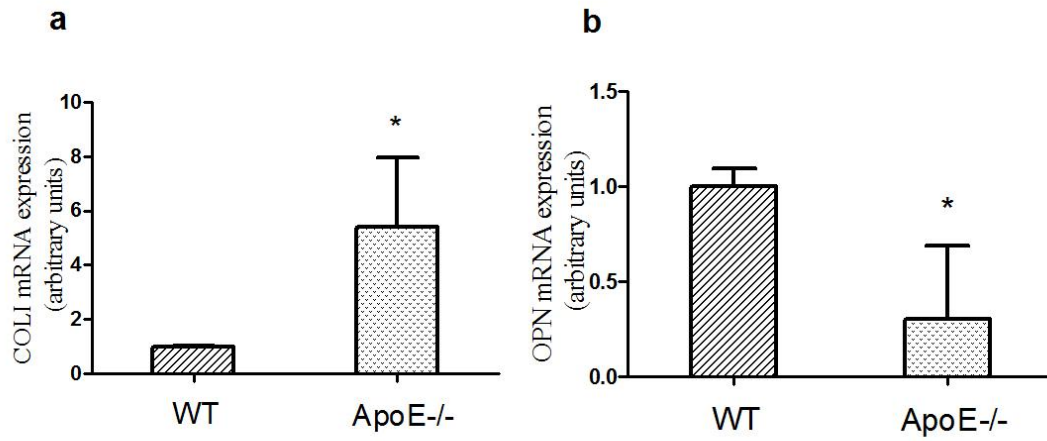


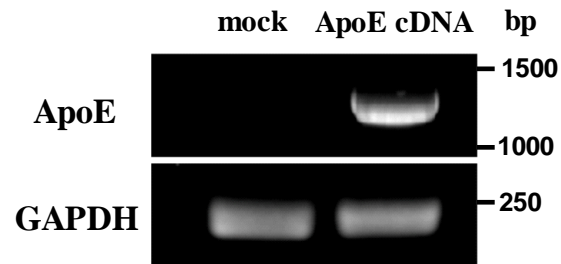
Figure 6. qRT-PCR analysis of osteogenic transcripts in WT and ApoE^{-/-} VSMCs.

Expression of COL1 and OPN mRNA was analyzed by qRT-PCR. **a**, qRT-PCR for COL1, and **b**, qRT-PCR for OPN. Data represents means \pm S.D. (n=3). * $p < 0.05$.

3. Overexpression of ApoE alleviates calcification in cultured VSMCs

To further confirm that ApoE regulates the calcification process, we reintroduced ApoE into ApoE^{-/-} VSMCs via plasmid transfection; these cells were designated as ApoE cDNA group. Empty vector transfected ApoE^{-/-} VSMCs were referred to as the mock group. Calcification experiments were performed on stably transfected cells. The existence of ApoE gene in VSMCs was confirmed by RT-PCR and western blot (Figure 7a and 7b). When incubated with 3.6mM Pi in medium, ApoE cDNA transfected VSMCs showed less calcification than the mock group, especially on day 6 and day 9 of treatment (Figure 8a-8i). ApoE deficient VSMCs express a lower basal protein level of SMC markers SM22 α and α SMA, as well as decreased mRNA expression of osteogenesis inhibitor OPN. A higher level of type I collagen mRNA was detected in ApoE^{-/-} VSMCs also. Runx2 protein levels were not significantly different between either ApoE^{-/-} and WT, or ApoE^{-/-} and ApoE cDNA transfected VSMC groups (Figure 9 and Figure 10), which is consistent with data obtained from ApoE^{-/-} and WT VSMCs.

a



b

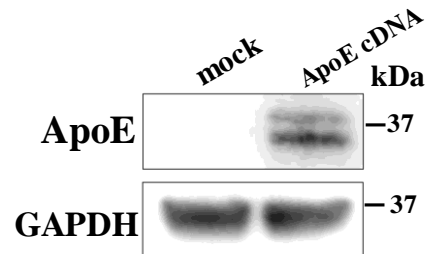
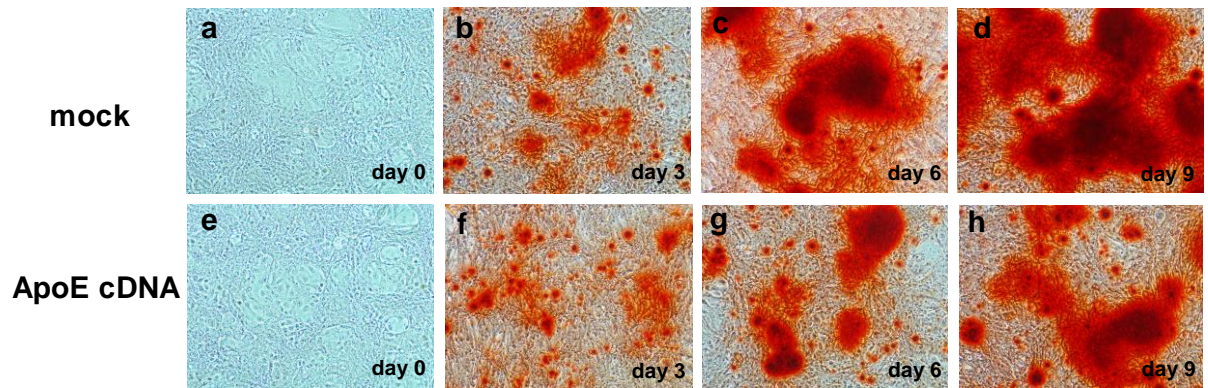


Figure 7. RT-PCR and Western blot analysis for ApoE expression in ApoE^{-/-} VSMCs transfected with murine ApoE cDNA. ApoE^{-/-} VSMCs were either transfected with ApoE cDNA or plasmid vector (mock) and incubated in selection medium for isolating successfully transfected cells. The expression of ApoE in transfected VSMCs was confirmed by RT-PCR (**a**) and western blot of the culture medium (**b**).



i

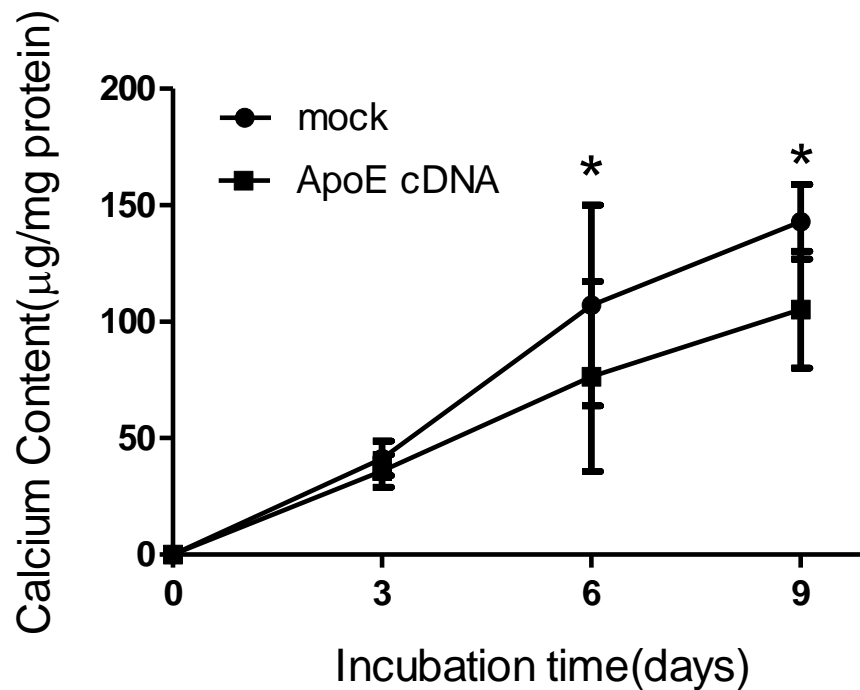


Figure 8. Calcification assays in ApoE^{-/-} VSMCs transfected with or without ApoE cDNA. Alizarin Red S staining of VSMCs was observed under microscope using a 10X objective. Calcification appeared as scattered deposits (**a-h**). Calcium assay was performed to quantify the calcium content in transfected VSMCs (n=4). The ApoE cDNA group of VSMCs showed significantly lower calcium content than mock cells on day 6 and day 9 of Pi treatment (**i**). * $p < 0.05$ vs. ApoE cDNA group.

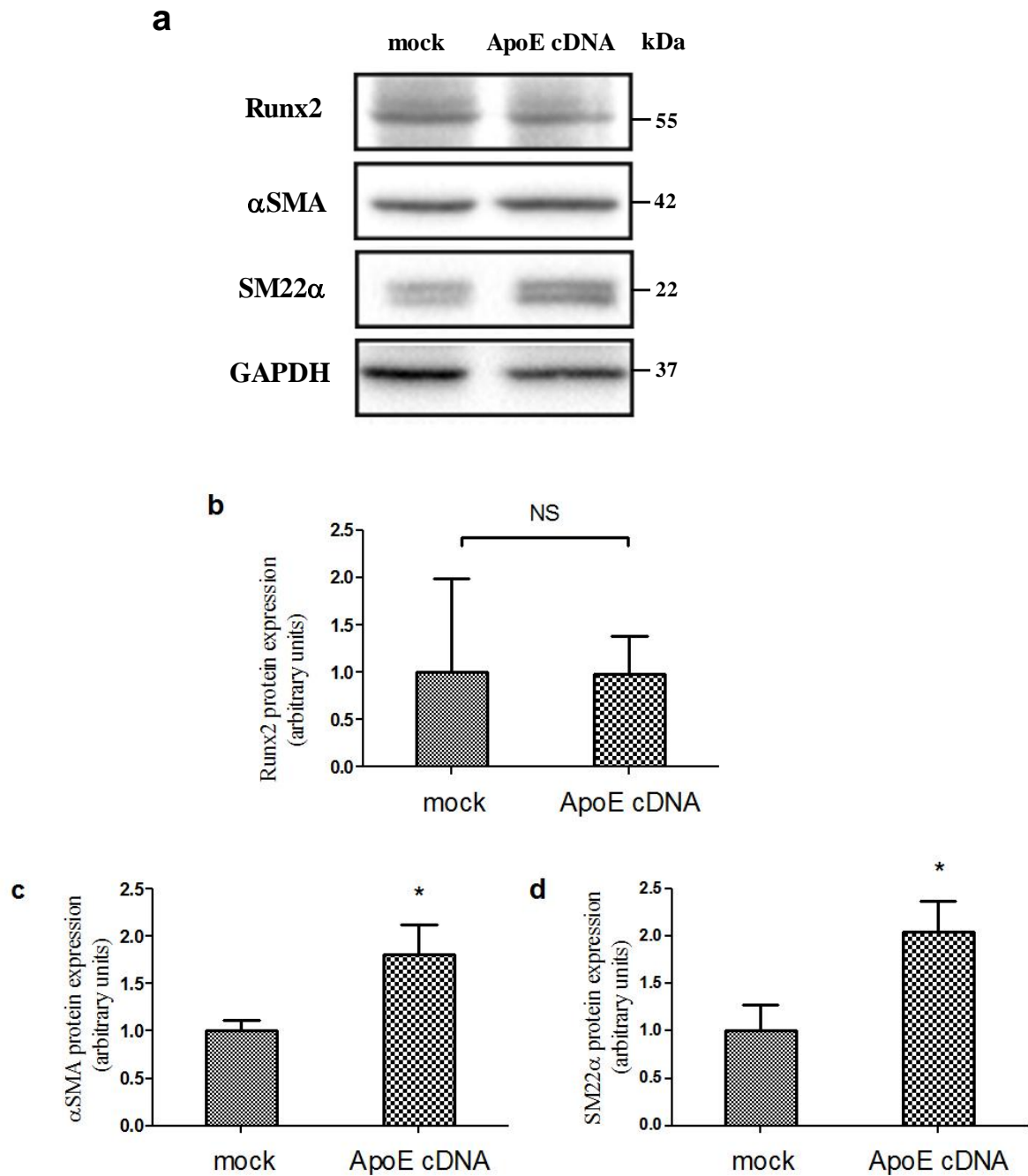


Figure 9. Western blot analysis of smooth muscle-specific(α SMA and SM22) and osteogenic (Runx2) biomarker in ApoE-transfected and mock VSMCs. ApoE^{-/-} VSMCs transfected with either vector (mock) or ApoE cDNA-expressing plasmid were collected. The protein expression of SM22 α , α SMA and Runx2 in the ApoE

cDNA transfected group and the mock group is detected by immunoblotting (**a**). Bars indicate fold change (n=3) determined by densitometry of target gene bands, normalized to GAPDH (**b-d**). * p<0.05.

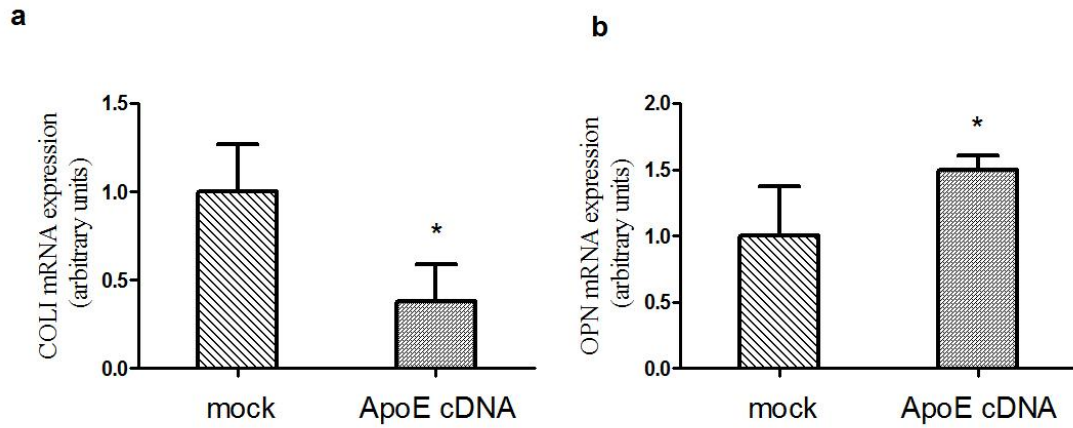


Figure 10. Analysis of the mRNA expressions of smooth muscle lineage markers and osteogenic genes in transfected VSMCs. Gene expressions of COLI and OPN on mRNA levels were analyzed by quantitative reverse transcription–polymerase chain reaction in ApoE^{-/-} VSMCs transfected with either vector (mock) or ApoE cDNA-expressing plasmid. **a.**qRT-PCR of COLI. **b.** qRT-PCR of OPN. Genes were indicated on the left side, and bars show normalized fold changes (n=3).* p<0.05.

4. Treatment with exogenous Apolipoprotein-E protein inhibits calcification of VSMC cultured from the aortas of ApoE^{-/-} mice

Our previous studies indicated that ApoE^{-/-} VSMCs are more susceptible to phosphate (Pi)-induced calcification than wildtype (WT) cells and the inhibitory effect on calcification of ApoE^{-/-} VSMCs was more obvious than that of the WT group (data not shown). Therefore, ApoE^{-/-} VSMCs was selected as the *in vitro* model to explore the function of ApoE during calcification. Accumulation of calcium deposits in ApoE^{-/-} VSMCs was detected by calcium assay after 9 days of incubation in calcification medium containing an elevated concentration of phosphate (3.6mM), compared with cells maintained in control medium. The addition of human apolipoprotein E (6µg/mL) into the culture medium significantly alleviated calcification in ApoE^{-/-} VSMCs (Pi vs. Pi+ApoE: 227.78±19.97 vs. 153.83±30.76 µg/mg, n=5, p<0.05) (Figure 11a). Alizarin S staining also shows positively-stained red areas in ApoE^{-/-} VSMCs under Pi treatment, which could not be seen in the control group of cells. ApoE, on the other hand, greatly shrank these areas (Figure 11b-11d).

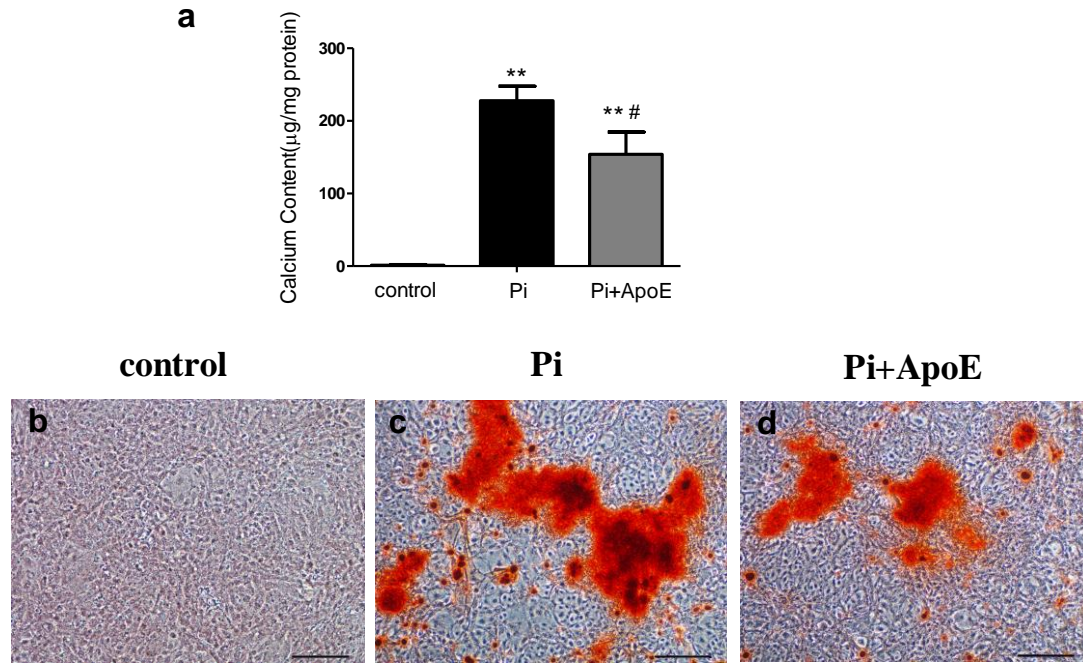


Figure 11. Analysis of calcification in VSMCs cultured from aorta tissues from ApoE^{-/-} mice. ApoE^{-/-} VSMCs were cultured in medium containing 3.6mM Pi for 9 days to mineralize. Colorimetric calcium assay was performed to quantify calcium level in untreated cells, cells treated with Pi, and cells treated with both Pi and ApoE protein (6 µg/mL)(a). Alizarin S staining was applied to visualize calcium deposits under microscope using a 10X objective (b-d). **p<0.01 vs. control.#p<0.05 vs. Pi.

5. Exogenous ApoE treatment inhibits osteoblast-like phenotype transformation of ApoE^{-/-} VSMCs

When induced to calcify, the protein level of smooth muscle marker SM22 α in ApoE^{-/-} VSMCs decreased, while Runx2 protein expression was enhanced, shown by western blot. ApoE attenuated these effects by decreasing the Runx2 protein level to control values, as well as inhibiting the loss of SM22 α during calcification ($p < 0.05$) (Figure 12). Besides, mRNA levels of calcification markers including alkaline phosphatase (ALP), Runx2, osteopontin (OPN) and osteocalcin (OCN) all increased greatly compared to the control group. ApoE protein dramatically reduced the expression of these osteogenesis-related genes in Pi treated VSMCs. Nevertheless, there was still a significant difference of ALP mRNA level between calcifying cells treated with ApoE and control cells ($p < 0.01$) (Figure 13), which suggests that ApoE failed to completely prevent the alternation of ALP as a response to Pi. These findings imply that ApoE is capable of partially reversing the osteoblast-like phenotypic change of VSMCs during calcification.

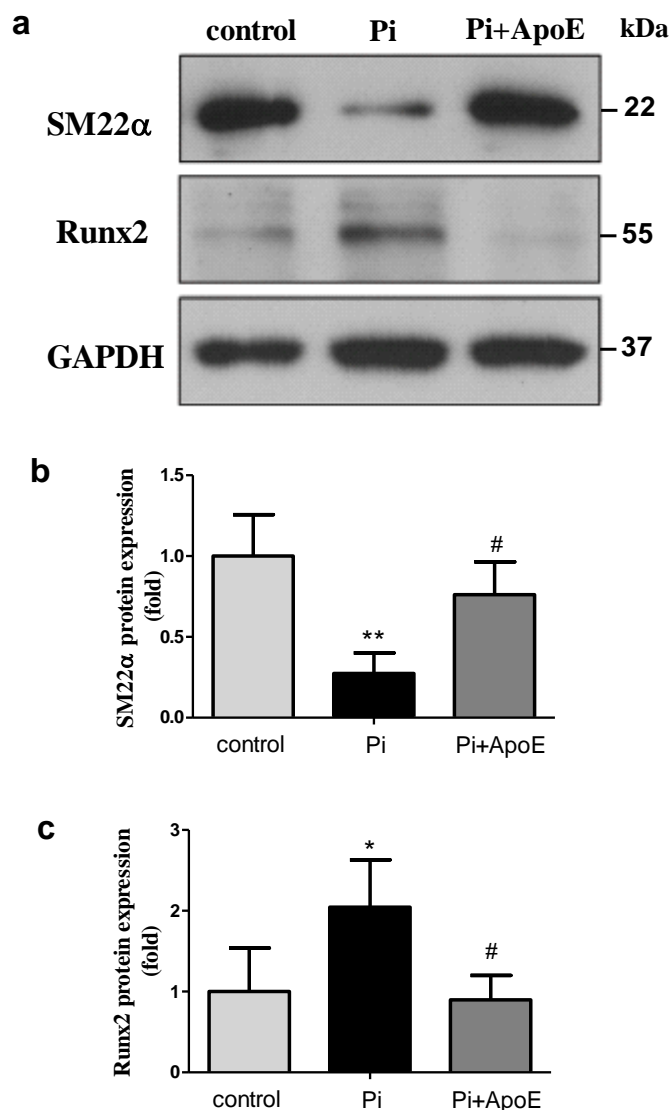


Figure 12. Western blot analysis of SM22 and Runx2 expression in ApoE^{-/-} VSMCs. Cells were incubated in regular control, calcification-inducing (3.6mM Pi), and Pi plus native human ApoE protein(6μg/mL) for 9 days, and then total proteins collected for Western blot analysis. **a-c**, Western blot for SM22α, Runx2 and GAPDH. A representative western blot is shown at the top. Bars indicate fold change (n=3) determined by densitometry of bands, normalized to GAPDH. * $p < 0.05$ vs. control. ** $p < 0.01$ vs. control. # $p < 0.05$ vs. Pi.

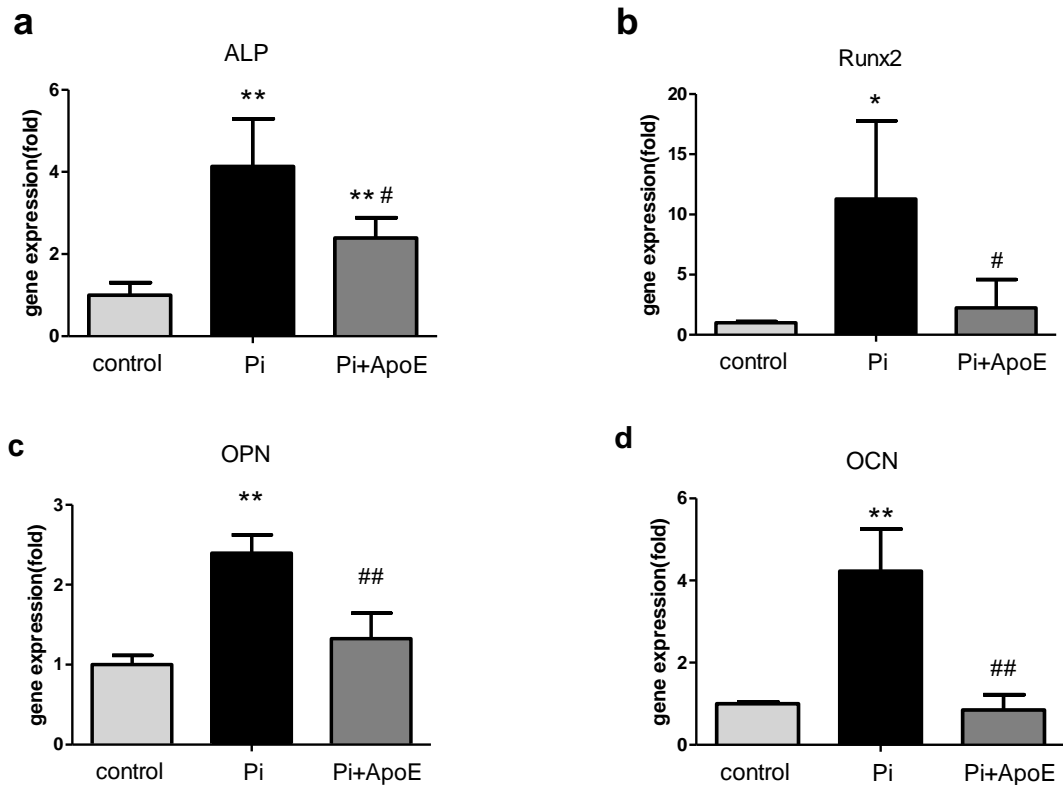


Figure 13. qRT-PCR analysis of calcification biomarkers in ApoE^{-/-}VSMCs with purified human ApoE protein in Pi-containing media. Cells were incubated either in regular medium or in calcification medium (3.6mM Pi) with or without ApoE protein (6μg/mL) for 9 days. mRNA expressions of calcification markers including ALP, Runx2, OPN and OCN were analyzed by quantitative reverse transcription-polymerase chain reaction(a-d). Bars indicate normalized fold change (n=5). ALP indicates alkaline phosphatase; OPN, osteopontin; OCN, osteocalcin.

*p<0.05 vs. control. **p<0.01 vs. control. #p<0.05 vs. Pi. ##p<0.01 vs. Pi.

6. The Wnt/ β -catenin pathway is involved in ApoE's attenuation of calcification

The activation of the canonical Wnt/ β -catenin pathway leads to the translocation of β -catenin into the cell nucleus. Western blotting was used to assess the presence of β -catenin in nuclear extracts from VSMCs. Incubation with Pi dramatically increased the amount of intranuclear β -catenin. However, when VSMCs were treated with Pi and ApoE together, expression of β -catenin in nuclear was suppressed (Figure 14a and 14b). Consistently, immunofluorescence revealed that β -catenin mainly resided in the cellular membrane and cytoplasm in control cells, while Pi treated VSMCs showed strong β -catenin staining in the nucleus. ApoE reduced the nuclear proportion of β -catenin, suggesting that ApoE inhibited canonical Wnt/ β -catenin pathway activation induced by Pi (Figure 14c). The involvement of Wnt/ β -catenin pathway was further confirmed by qRT-PCR of β -catenin target genes, including Axin2, CyclinD1 and Tcf4 (Figure 15), which were all increased during calcification ($p < 0.05$). However, Pi failed to stimulate the expression of these genes to rise when cells were subject to ApoE treatment. To clarify whether ApoE still protects ApoE^{-/-} VSMCs from calcification under conditions in where Wnt/ β -catenin pathway is constitutively activated, we used the canonical β -catenin activator Wnt3a to treat cells. Under the calcifying condition, VSMCs exhibited a 1.4-fold increase in mineralization level when Wnt3a was added into the medium (Pi vs. Pi+Wnt3a: 176.45 ± 30.21 vs. 249.35 ± 61.72 $\mu\text{g}/\text{mg}$, $n=4$, $p < 0.05$); ApoE lost the ability to

attenuate calcification in the presence of Wnt3a. Alizarin Red S also showed enlarged areas of positive staining in Wnt3a treated VSMCs, regardless of whether ApoE was applied to cells or not (Figure 16).

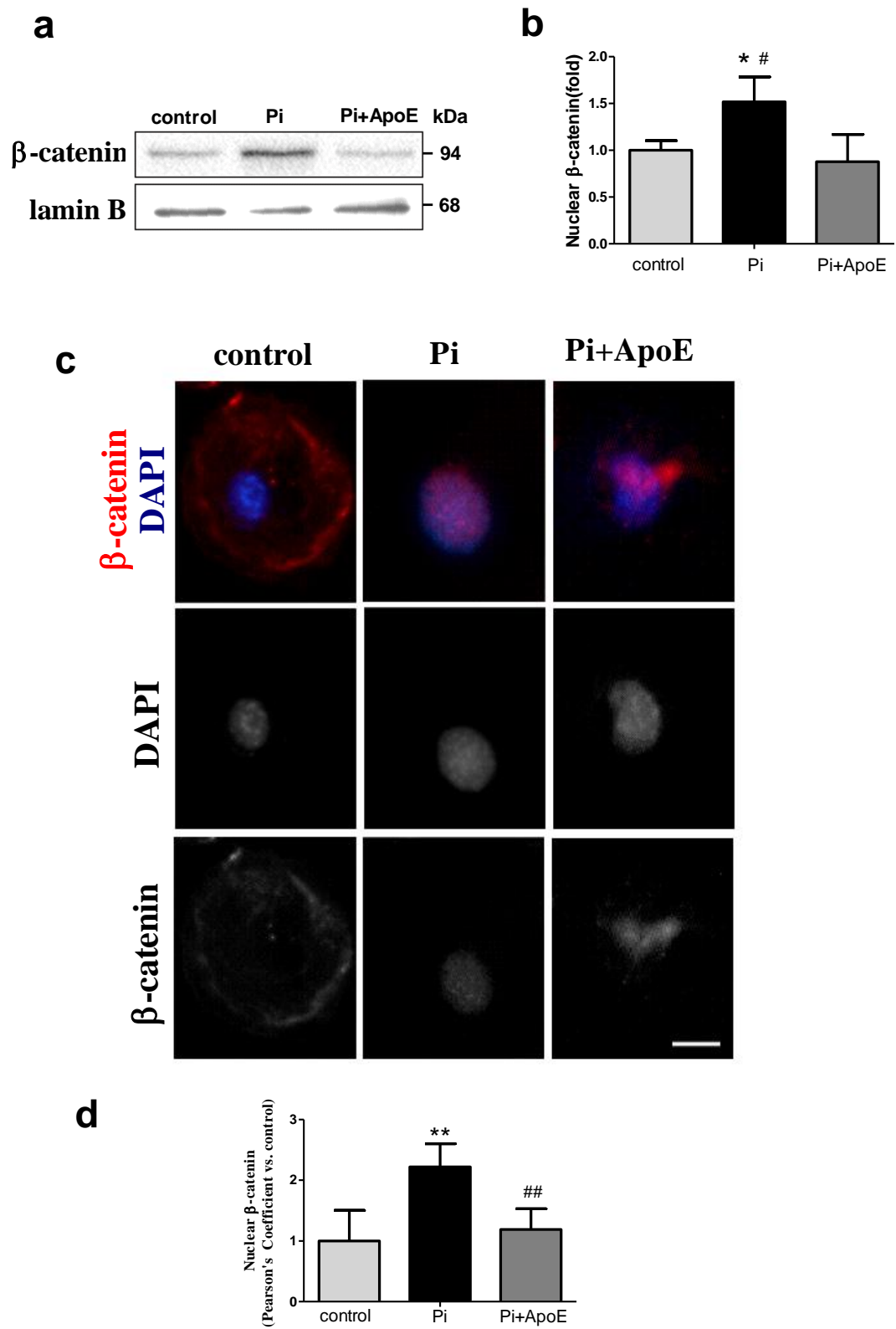


Figure 14 . Western blot and immunofluorescence analysis of β -catenin expression in ApoE^{-/-} VSMCs treated with human ApoE. Cells were grown in regular medium or in calcification medium (3.6mM Pi) with or without ApoE protein (6 μ g/mL) for 24 hours. **a** and **b**, Western blot using nuclear protein extracts from VSMCs. A representative western blot is shown at the top. Bars indicate fold change (n=3) determined by densitometry of bands, normalized to lamin B. **c**, Subcellular localization of β -catenin (red) visualized by immunofluorescence. Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI)(blue). Scale bar=2.5 μ m. **d**, Cells from 3 random fields under each condition were scored for β -catenin localization and analyzed by the Pearson's coefficient. Bars indicate fold changes.

****p<0.01 vs. control.##p<0.01 vs. Pi.**

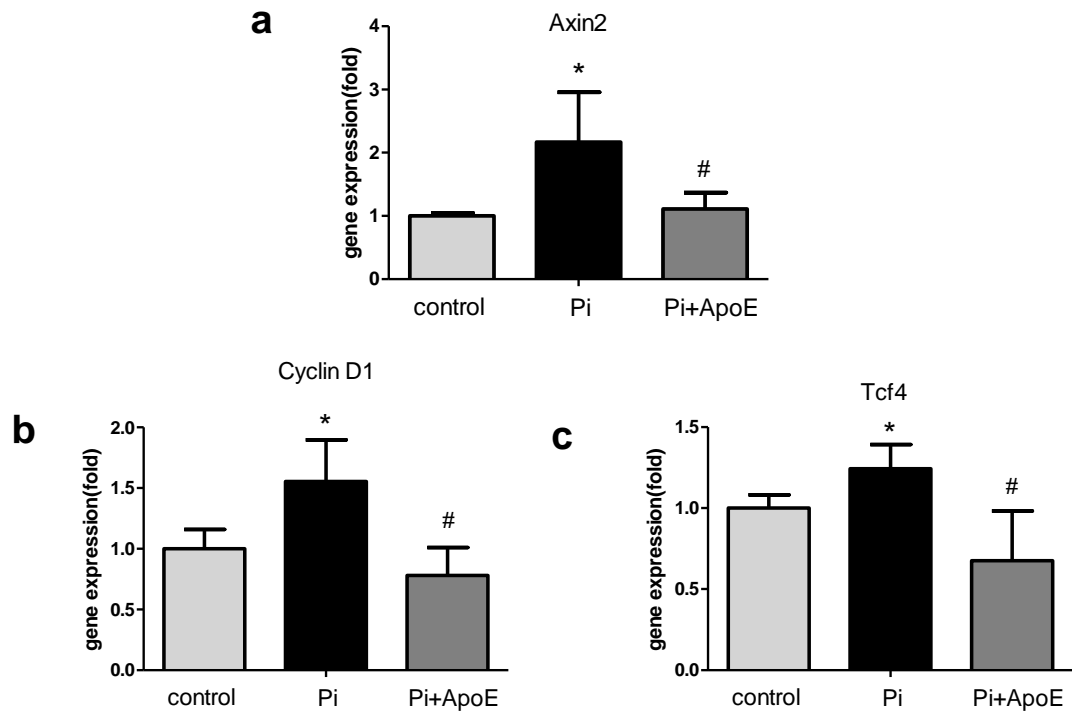


Figure 15. qRT-PCR assessment of β -catenin target gene expression in ApoE^{-/-} VSMCs. Cells were incubated either in regular medium or in calcification medium (3.6mM Pi) with or without ApoE protein (6 μ g/mL) for 24 hours. **a**, Axin2; **b**, Cyclin D1; and **c**, Tcf4. Data represents means \pm S.D., n=5. * p <0.05 vs. control; and # p <0.05 vs. Pi.

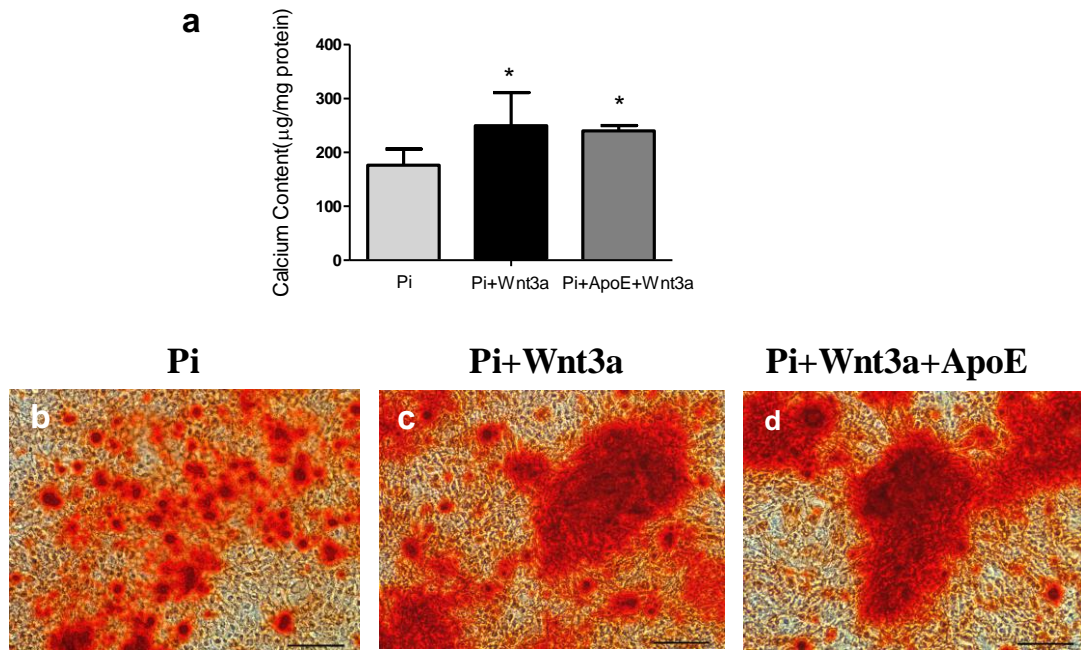


Figure 16. Calcification of VSMCs treated with Wnt3a and/or ApoE protein.

ApoE^{-/-} VSMCs were incubated either in calcification medium (3.6mM Pi) or in calcification medium with Wnt3a (20ng/mL) and/or ApoE protein (6µg/mL) for 9 days **a**, Calcium assay of calcium deposits in VSMCs (n=4). **b-d**, Alizarin Red S staining was observed under microscope using a 10X objective. *p<0.05 vs. Pi.

7. ApoE deficiency attenuates expression of GTAP in murine aortas and aorta-derived VSMCs.

The expression of GTAP in ApoE^{-/-} and WT aortas and VSMCs was detected by Western blot. Protein was extracted from either murine cells or aortas. A stronger signal of GTAP was detected in WT aortas than in ApoE^{-/-} ones (Figure 17a), which indicates that wild type mice generally have higher protein expression of GTAP in aortas than ApoE^{-/-} mice. Similarly, aortic smooth muscle cells that are ApoE-deficient exhibit lower GTAP expression than WT VSMCs (Figure 17b).

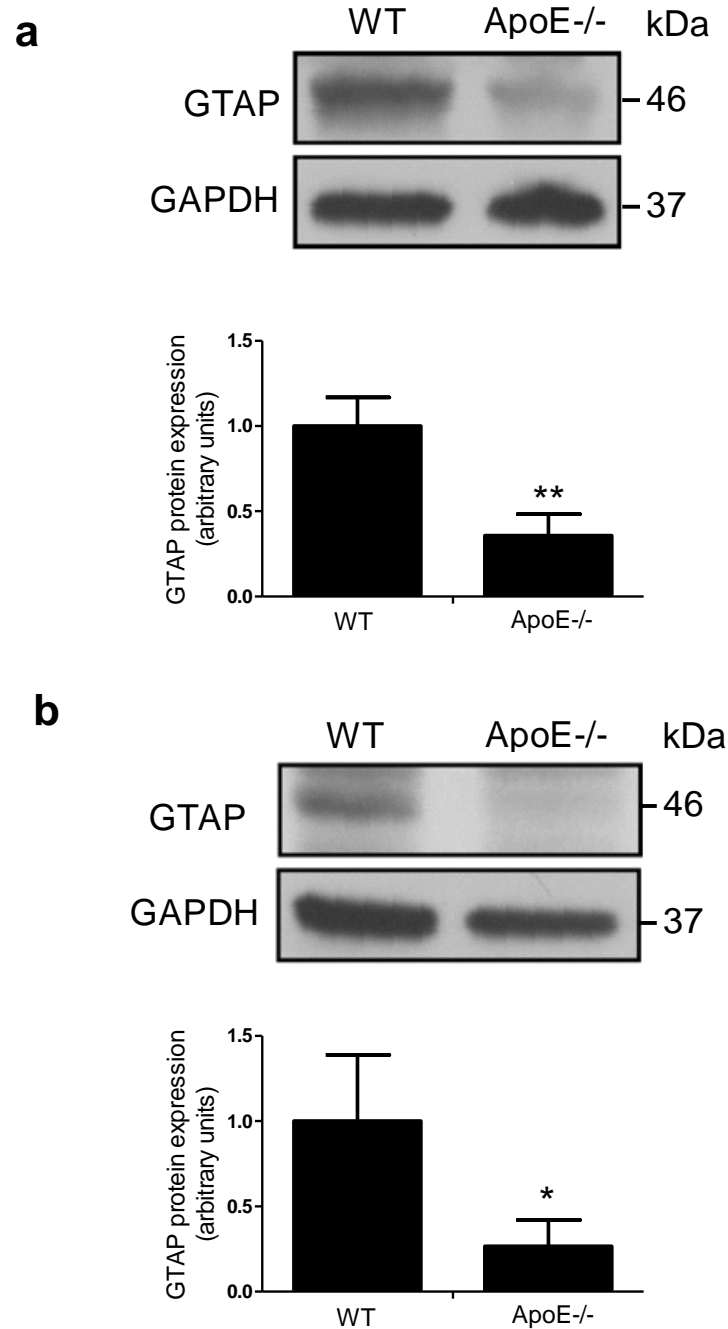


Figure 17. Western blot analysis of GTAP expression in murine aortas and aorta-derived VSMCs. Total proteins extracted from aortic tissues and VSMC cultured from aortas were used to assess the protein level of GTAP by Western blot. **(a)** Western blot for GTAP and GAPDH in aortic tissues; and **(b)** Western blot for GTAP

and GAPDH in VSMC cultures. Bars represent means \pm SD; n = 4 per group for aortas and n=3 for VSMCs. *p<0.05 vs. WT, **p<0.01 vs. WT.

8. Human plasma ApoE induces GTAP in VSMCs from ApoE^{-/-} but not wild type mice.

Both ApoE^{-/-} and WT VSMCs were treated with 6µg/mL human ApoE for up to 4 hours, after 24 hours starvation in serum-free medium. Western blot was performed to detect GTAP expression level. WT VSMCs showed a clear band of the predicted size of GTAP (46kDa), even in cells without ApoE treatment, indicating that WT VSMCs have a potent expression of GTAP protein. Moreover, GTAP band intensity in WT VSMCs did not change significantly after ApoE treatment for up to 4 hours, which suggests that the level of GTAP was not dramatically affected by ApoE in WT VSMCs (Figure 18a). ApoE^{-/-} VSMCs, on the other hand, exhibited barely detectable GTAP protein expression at the absence of ApoE in culture medium. In ApoE^{-/-} VSMCs treated with ApoE, GTAP expression increased in a time-dependent manner (Figure 18b); a statistically significant difference was found between untreated cells and cells treated with ApoE for 2 hours or 4 hours.

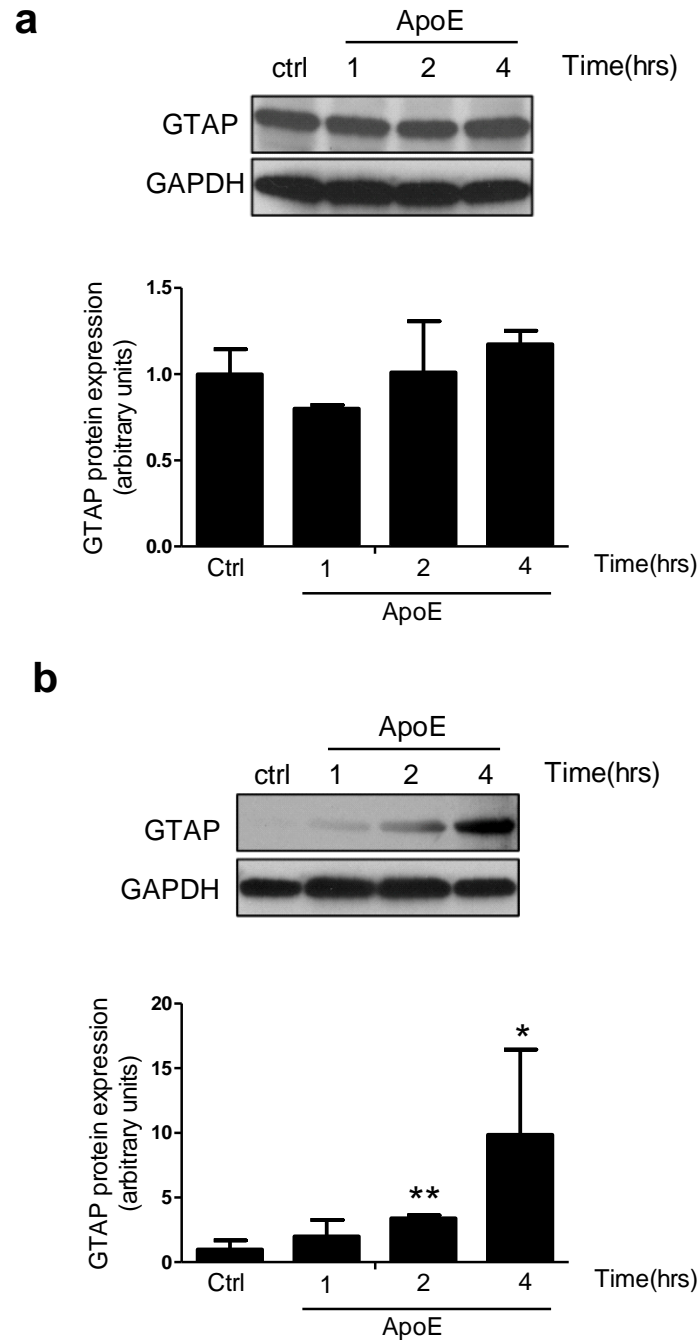


Figure 18. Western blot analysis of GTAP expression in wild type and ApoE-null VSMCs treated with or without purified human ApoE protein. VSMCs were incubated with DMEM control medium (Ctrl) or with 6 μ g/mL ApoE for up to 4 hrs. **a**, WT VSMCs; and **b**, ApoE^{-/-} VSMCs. GTAP band intensity was normalized to

GAPDH. Bars represent means \pm S.D; n = 3 per group. *p<0.05 vs.ctrl, **p<0.01 vs.
Ctrl.

9. GTAP decreases in wildtype (WT) VSMCs during calcification

To determine whether GTAP expression alters in the process of calcification, either WT or ApoE^{-/-} VSMCs were treated with 3.6mM Pi for up to 9 days. Cells were collected at various time points and Western blot was performed to detect GTAP expression. Interestingly, in WT VSMCs, the intensity of GTAP protein band decreased in a time-dependent manner; densitometry together with statistical analysis showed GTAP expression in cells incubated with 3.6mM Pi was significantly lower than cells cultured in regular medium(Figure 19a). In ApoE^{-/-} VSMCs, GTAP levels almost remained unchanged 3 days, 6 days, and 9 days after treatment with Pi started ($p>0.05$)(Figure 19b).

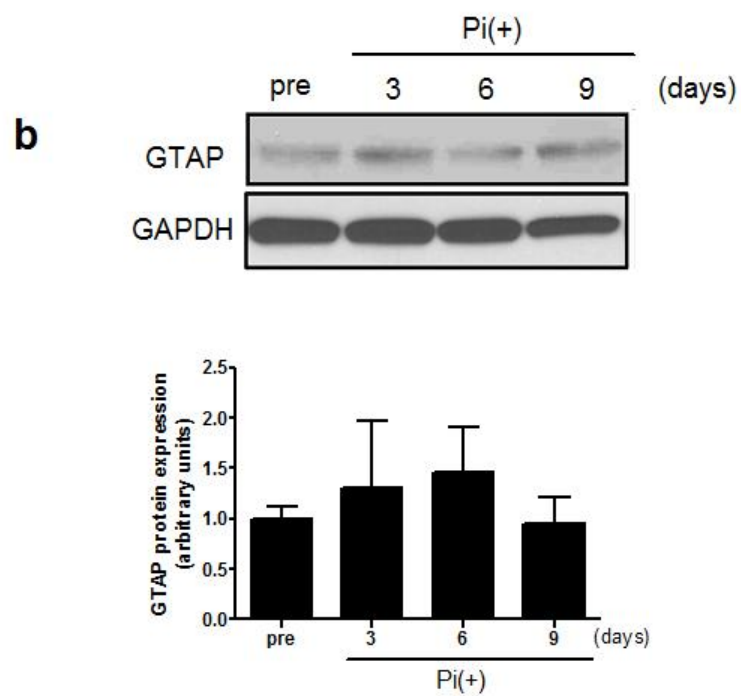
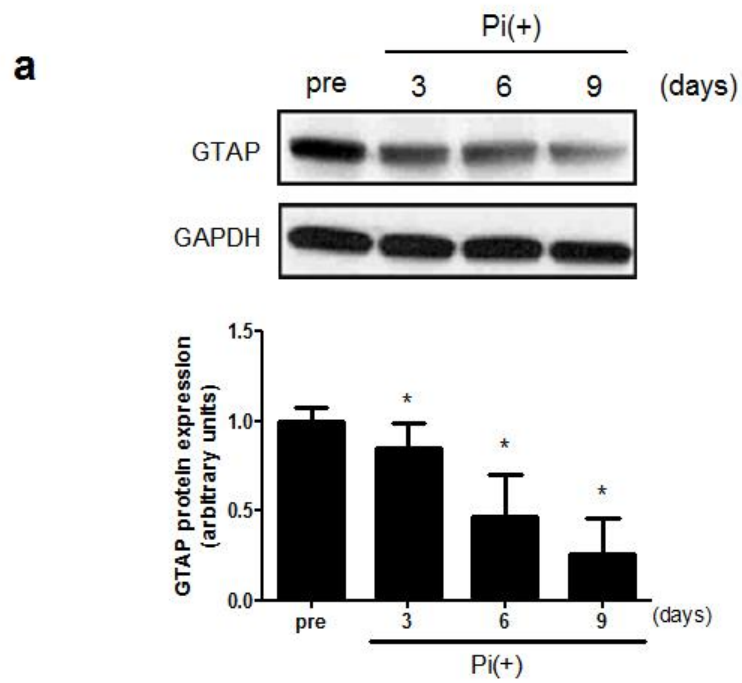


Figure 19. Western blot analysis of GTAP in VSMCs incubated with Pi. Wildtype or ApoE^{-/-} VSMCs were incubated with 3.6mM Pi for up to 9 days. Total proteins extracted from VSMC cultured from aortas were used to assess the protein level of GTAP by Western blot in WT (**a**) and ApoE^{-/-} (**b**) VSMCs. Bars represent means \pm SD; n = 3 per group. *p<0.05 vs.pre.

10. Calcification in VSMCs that overexpress GTAP

Lentiviral expression plasmid CD513B-1 that contains 3XFLAG GTAP cDNA was constructed for transduction of VSMCs. Because GTAP functions as an E2 ubiquitin-conjugating enzyme thorough the active cysteine amino acid residue (C351) in the C terminus, another construct that expresses GTAP with the active cysteine mutated to alanine was used as well to study the relationship of GTAP-mediated ubiquitination and calcification. CD513B-1 also encodes green fluorescence protein (GFP) and puromycin selection marker. Both WT and ApoE^{-/-} were infected with lentiviral particles and incubated in selective culture medium for 3 weeks in order to obtain cells stably overexpressing GTAP. Cells were observed under a fluorescent microscope and green fluorescence was detected in infected WT and ApoE^{-/-} VSMCs (Figure 20a-20f), which indicates successful infection. In both cells, the vector group showed the strongest fluorescence among all the groups. Cells overexpressing wildtype GTAP (designated as GTAP^{WT}) had a weaker signal compared to cells overpressing mutated GTAP (designated as GTAP^{C351A}). Expression of 3XFLAG GTAP was confirmed by Western blot (Figure 20g and 20h).

Then VSMCs were treated with 3.6mM Pi for 9 days to induce calcification. In WT VSMCs, overexpression of GTAP^{WT} or GTAP^{C351A} decreased calcification compared to vector group; GTAP^{WT} showed more dramatic suppression of cellular mineralization than GTAP^{C351A} group, reflected by Alizarin Red S staining (Figure

21a-21c) and calcium assay (Figure 21d). In ApoE^{-/-} VSMCs, no significant difference in the level of calcification was found between vector, GTAP^{WT} and GTAP^{C351A} groups of cells in response to Pi incubation (Figure 21e-21h).

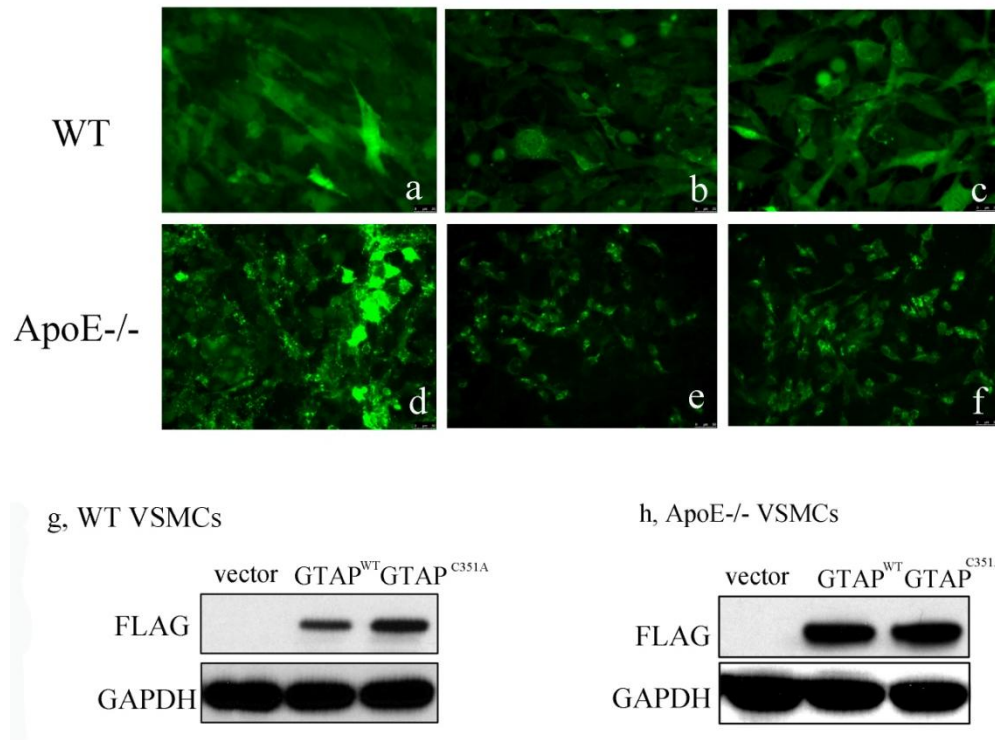
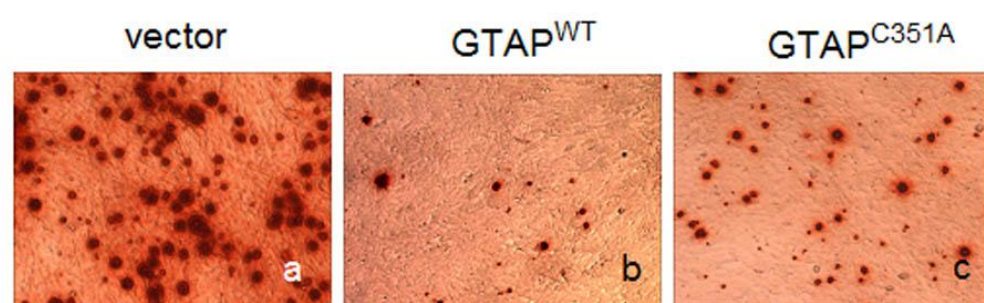
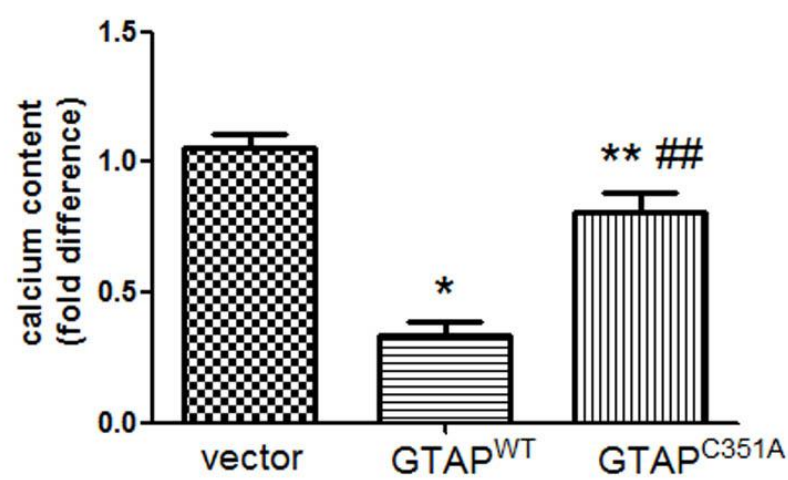


Figure 20. Immunofluorescence and Western blot analysis of VSMC infected by GTAP-expressing lentivirus. Immunofluorescence images of wildtype (a-c) and ApoE^{-/-} (d-f) VSMCs infected by lentivirus that express vector only, wildtype GTAP (GTAP^{WT}), or mutated GTAP (GTAP^{C351A}) were observed under fluorescence microscope (400X). Western blot was performed on lysis of WT (g) and ApoE^{-/-} (h) VSMCs. GAPDH was used as the loading control.



d



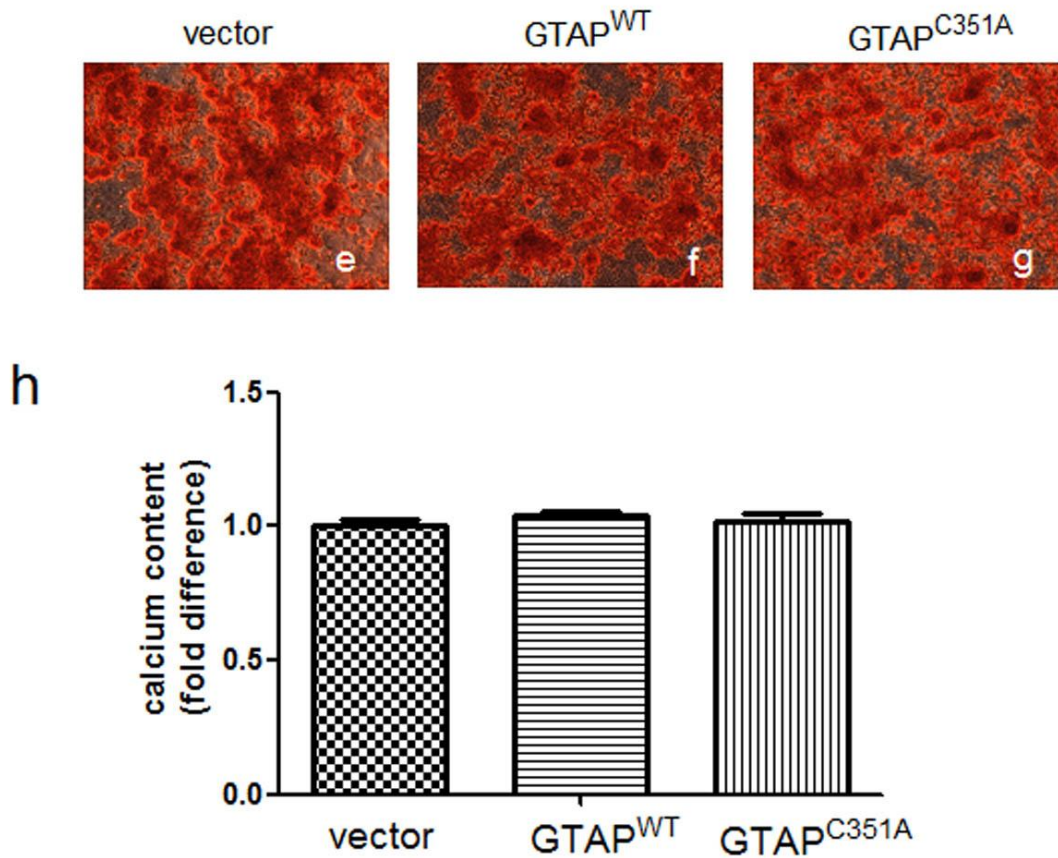


Figure 21. Assays for calcification in VSMCs infected with wild type and mutant GTAP. Wildtype(WT) or ApoE^{-/-} VSMCs that overexpress wildtype GTAP (GTAP^{WT}) or mutant GTAP (GTAP^{C351A}) were incubated with 3.6mM Pi for 9 days. Alizarin Red S staining of WT (a-c) and ApoE^{-/-} (e-g) VSMCs were performed to visualize calcium deposits, which were observed under microscope using a 10X objective. Calcium assay of WT (d) and ApoE^{-/-} VSMCs (h) were applied to quantify calcium content. Bars represent means \pm SD; n = 3 per group. *p<0.05 vs.vector.**p<0.01vs. vector. ##p<0.01 vs.GTAP^{WT}.

11. Increased calcification in the aortic tissue of GTAP^{-/-} mice

Aortic tissues were dissected from 8-10 weeks old male mice of 129sv background. Aortas were collected from wildtype 129sv mice (GTAP^{+/+}), heterozygous GTAP-deficient mice (GTAP^{+/-}) and GTAP knockout mice (GTAP^{-/-}). Part of aortic tissues were lysed and used for Western blot; GTAP protein expression level was assessed in these three groups of samples. There was barely detectable GTAP in aortic tissues from GTAP^{-/-} animals, while GTAP^{+/-} and GTAP^{+/+} mice showed visible GTAP band. The expression of GTAP in GTAP^{+/+} aortas was more potent than GTAP^{+/-} ones (Figure 22a). Meanwhile, some aortic tissues were cut into aortic rings and placed onto plates. Culture medium with 2.6mM Pi was used to induce calcification of aortic rings for 9 days. After treatment, calcium assay was performed to assess calcification, standardized to dry weights of aortic rings. Aortic rings from GTAP^{-/-} mice showed significantly higher calcium content than aortic rings from GTAP^{+/+} or GTAP^{+/-} mice, suggesting GTAP deficiency renders aortic rings more susceptible to Pi-induced calcification. No obvious difference was observed in calcification between GTAP^{+/+} and GTAP^{+/-} aortic rings (Figure 22b).

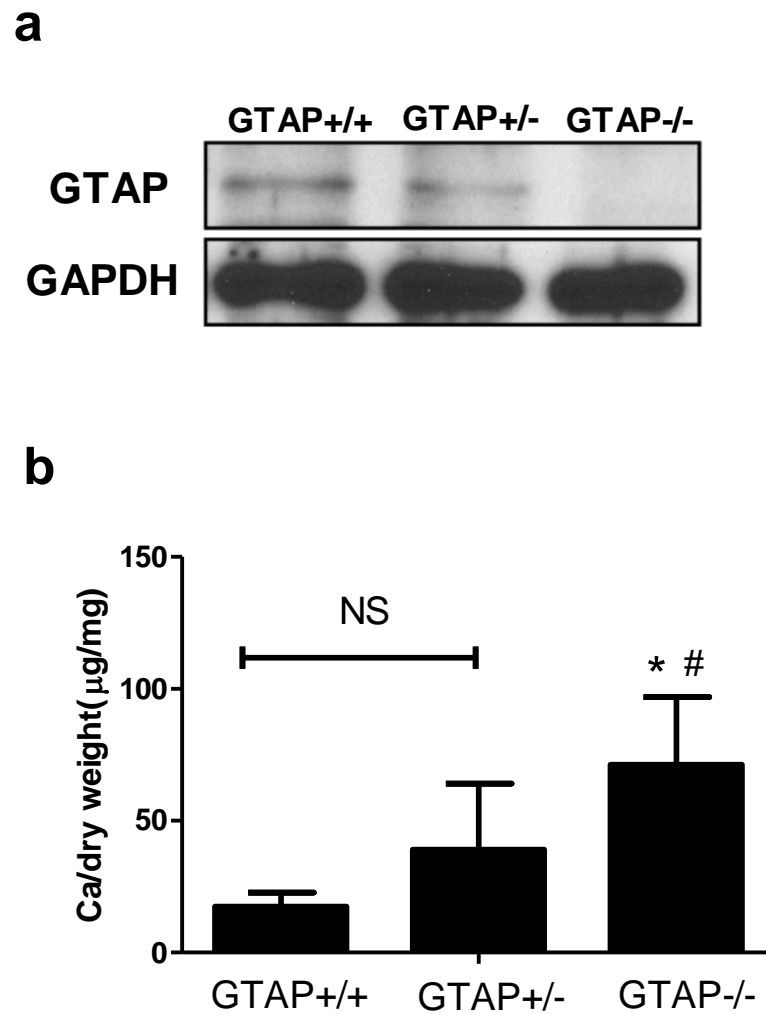


Figure 22. Western blot analysis of GTAP expression and calcification assays in murine aortic rings incubated with Pi *ex vivo*. Aortas were dissected from wildtype 129sv mice (GTAP^{+/+}), heterozygous GTAP-deficient mice (GTAP^{+/-}) and GTAP knockout mice (GTAP^{-/-}). Aortas were either collected for immunoblotting or cut into rings and incubated in calcification medium (2.6mM Pi) for 9 days. **a.** Western blot analysis of GTAP expression in aortas without Pi treatment. **b.** Calcification of murine aortic rings in response to elevated phosphate, bars represent means \pm SD; n=4 per group. *p<0.05 vs. GTAP^{+/+}. #p<0.05 vs. GTAP^{+/-}.

12. Decreased expression of GTAP in VSMCs exacerbates calcification

In order to knock down GTAP in VSMCs, a pLKO.1 lentiviral expression vector that contains a sequence which targets GTAP was constructed. Lentiviral particles were produced by transfection of 293ft cells with expression vector together with packaging plasmids. VSMCs were infected with virus expressing either negative shRNA that does not target any known sequence in murine genome, or GTAP shRNA. To achieve stable lentivirus-delivered RNA interference (RNAi), VSMCs were selected by the addition of puromycin and GTAP expression was analyzed by Western blot (Figure 23a). Compared to cells infected with negative shRNA control, GTAP was drastically reduced in cells infected with lentivirus expressing GTAP shRNA. Then infected VSMCs were incubated with 3.6mM Pi to induce calcification. Level of calcification was assessed by Alizarin Red S staining (Figure 23b and 23c) and calcium assay (Figure 23d). In GTAP knockdown VSMCs, more positive Alizarin Red S staining was observed in cell culture and higher calcium content was detected, which indicates inhibited expression of GTAP worsened calcification in VSMCs treated with elevated level of Pi.

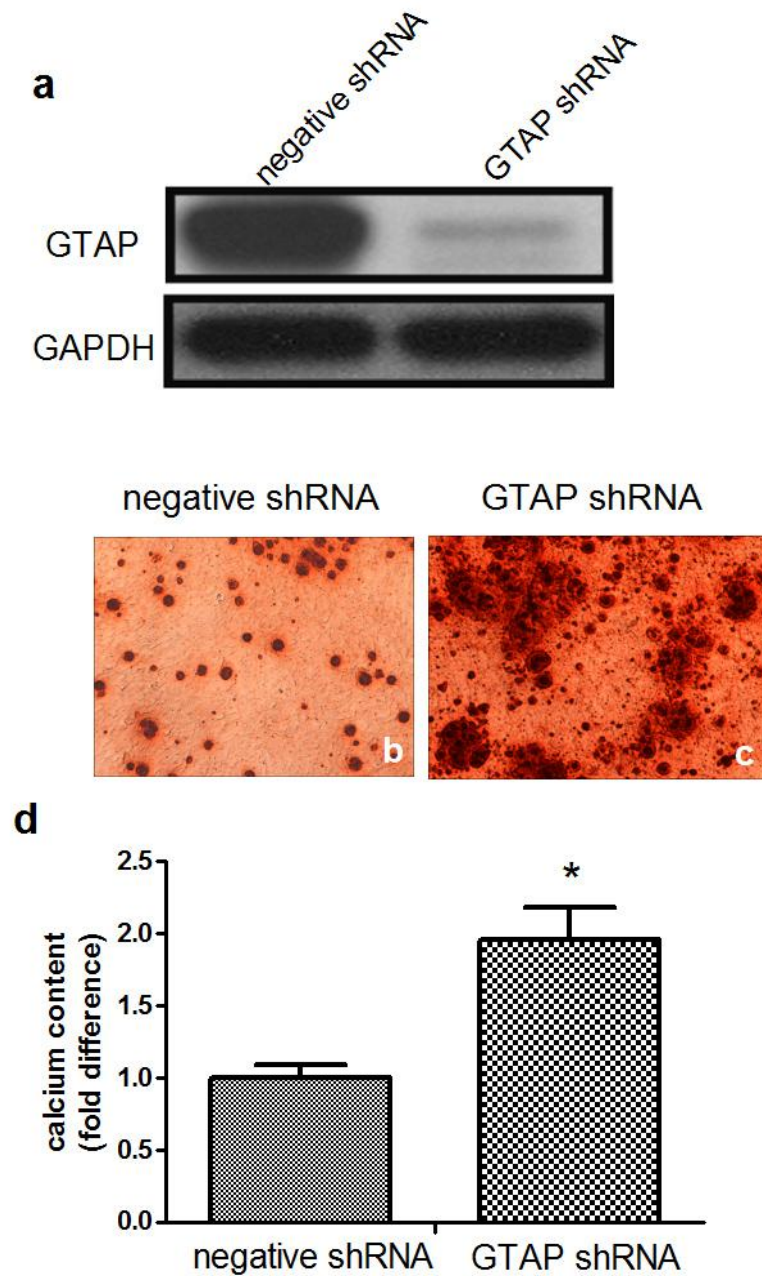


Figure 23. Western blot analysis of GTAP and calcification assays in murine VSMCs treated with shRNA. Wildtype VSMCs infected with lentivirus that express either negative shRNA or GTAP shRNA were treated with 3.6mM Pi to induce

calcification for 9 days. **a.**Before Pi treatment, western blot analysis of GTAP expression in VSMCs that express negative shRNA or GTAP shRNA was performed to confirm the knockdown of GTAP protein level. Alizarin Red S staining (**b** and **c**) and calcium assay (**d**) of GTAP knockdown VSMCs after incubation with 3.6mM Pi for 9 days. Bars represent means \pm SD; n=3 per group.*p<0.05 vs. negative shRNA.

13. Increased expression of GTAP upregulates smooth muscle markers in VSMCs

Wildtype VSMCs that stably express lentiviral vector only or lentiviral vector that contains either GTAP^{WT} or GTAP^{C351A} cDNA were collected and Western blot was performed to detect expressions of osteogenetic gene Runx2, as well as smooth muscle lineage markers α SMA and SM22 α . VSMCs that overexpress GTAP^{WT} expressed higher levels of α SMA and SM22 α than VSMCs that express vector. Overexpression of GTAP^{C351A} enhanced, slightly but not significantly, expression of α SMA in VSMCs. The expression of SM22 α was elevated in VSMCs expressing GTAP^{WT} or GTAP^{C351A}, in comparison to vector group. A weaker signal of Runx2 was detected when cells overexpress GTAP^{WT} (Figure 24).

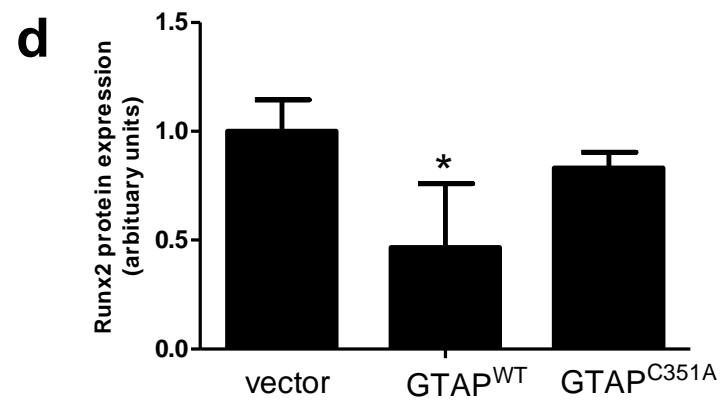
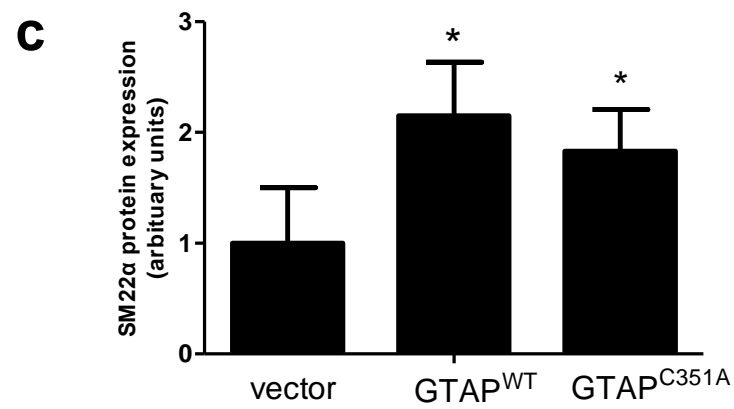
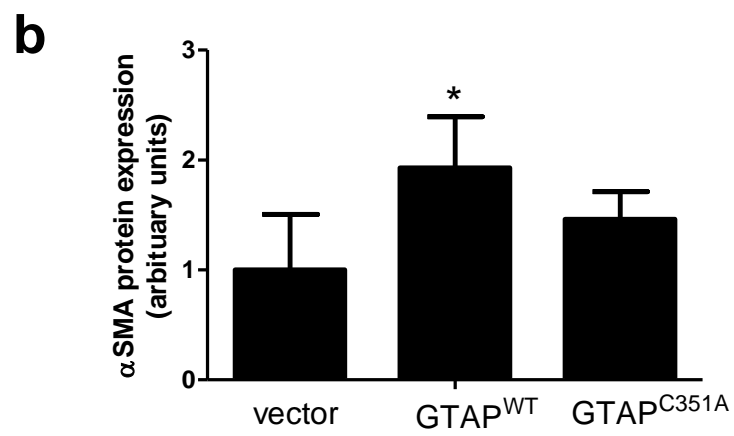
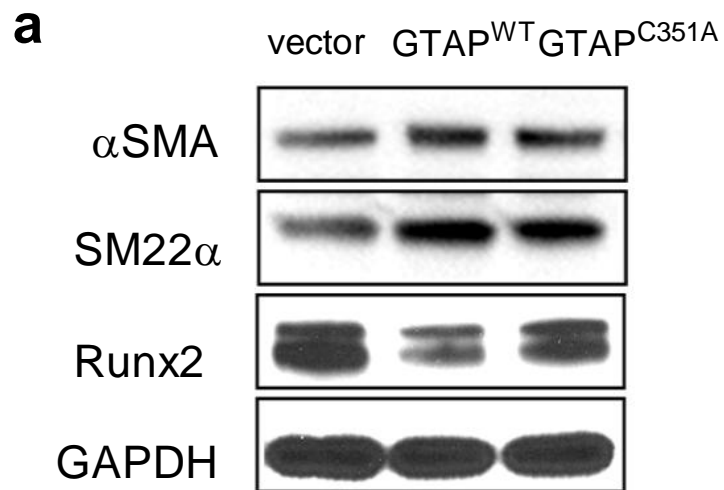


Figure 24. Western blot analysis of smooth muscle and osteogenic lineage biomarkers in VSMCs transduced with wild type and mutant GTAP. VSMCs that expresses wildtype GTAP(GTAP^{WT}) or mutated GTAP(GTAP^{C351A}) via lentiviral infection were lysed and Western blot was performed. VSMCs expressing vector were used as the control. **a.** A representative Western blot of α SMA, SM22 α and Runx2 is shown on top. **b.** Bars indicate fold change determined by densitometry of target gene bands, normalized to GAPDH. n=3 per group. *p<0.05 vs. vector.

14. Decreased expression of GTAP downregulates smooth muscle markers in VSMCs.

Wildtype VSMCs that stably express negative shRNA which does not target any known sequence in murine genome or GTAP shRNA via lentiviral infection were lysed and Western blot was performed. Expressions of smooth muscle lineage markers α SMA and SM22 α were downregulated in VSMCs expressing GTAP shRNA compared to negative shRNA-expressing cells. However, knockdown of GTAP with lentiviral shRNA greatly enhanced the expression of Runx2, the master regulator of osteogenesis (Figure 25).

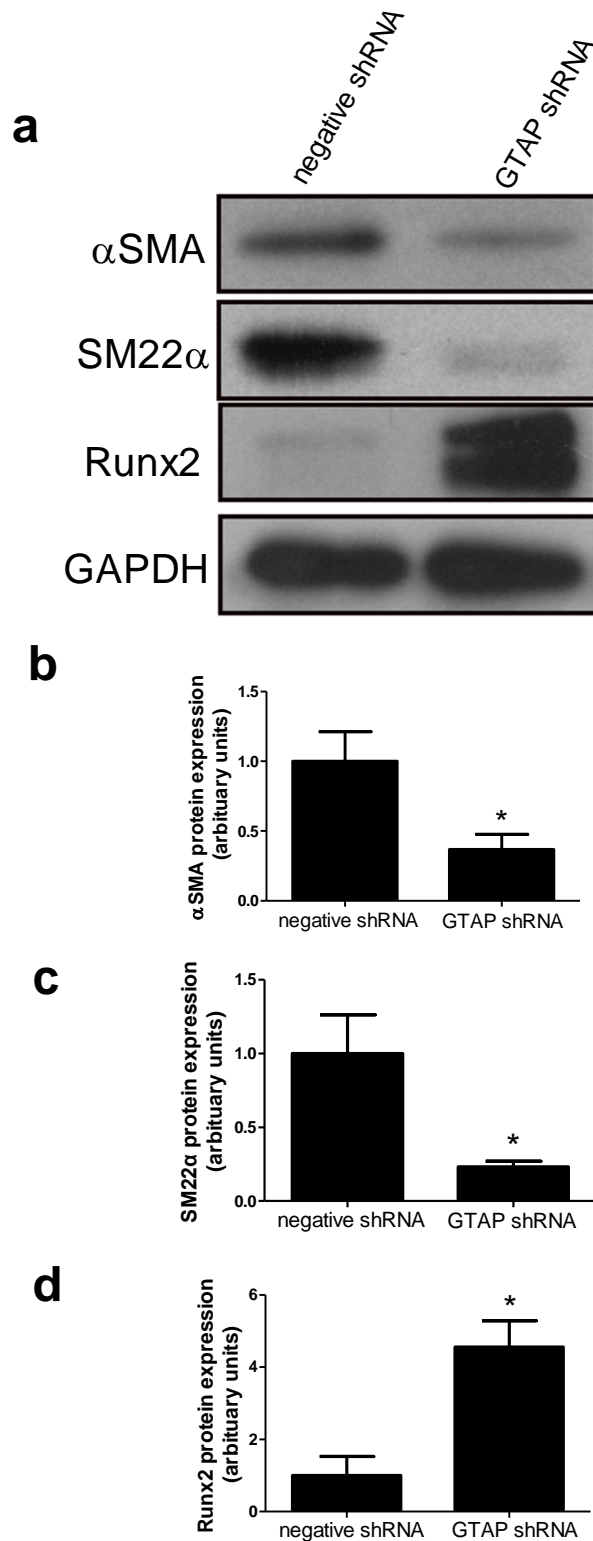


Figure 25.Analysis of expressions of smooth muscle lineage markers and osteogenic gene in GTAP knockdown VSMCs. VSMCs that expresses negative shRNA or GTAP shRNA via lentiviral infection were lysed and Western blot was

performed. **a.** A representative Western blot of α SMA, SM22 α and Runx2 is shown on top. **b.** Bars indicate fold change determined by densitometry of target gene bands, normalized to GAPDH. n=3 per group. *p<0.05 vs. negative shRNA.

15. Augmentation of Runx2 expression by knockdown of GTAP with GTAP-specific shRNA

To determine the role of GTAP in Runx2 degradation, VSMCs that stably express either negative shRNA controls or GTAP shRNA were incubated with cycloheximide (CHX) to inhibit protein synthesis for up to 4 hours. Western blot analysis of total proteins extracted from VSMCs with or without shRNA suppression of GTAP (Figure 26) showed different patterns of Runx2 protein expression. In control cells with negative shRNA, Runx2 declined gradually with prolonged incubation with CHX, suggesting that Runx2 pre-existing in the cells was in the process of degradation. However, when GTAP was knockdown in VSMCs with specific shRNA, the levels of Runx2 expression only decreased slightly during the entire 4-hour period of time, compared to control cells. This result manifests that in the cells with shRNA-mediated decreased expression of GTAP, half-life of Runx2 is prolonged, which suggests the degradation of Runx2 depends on GTAP expression.

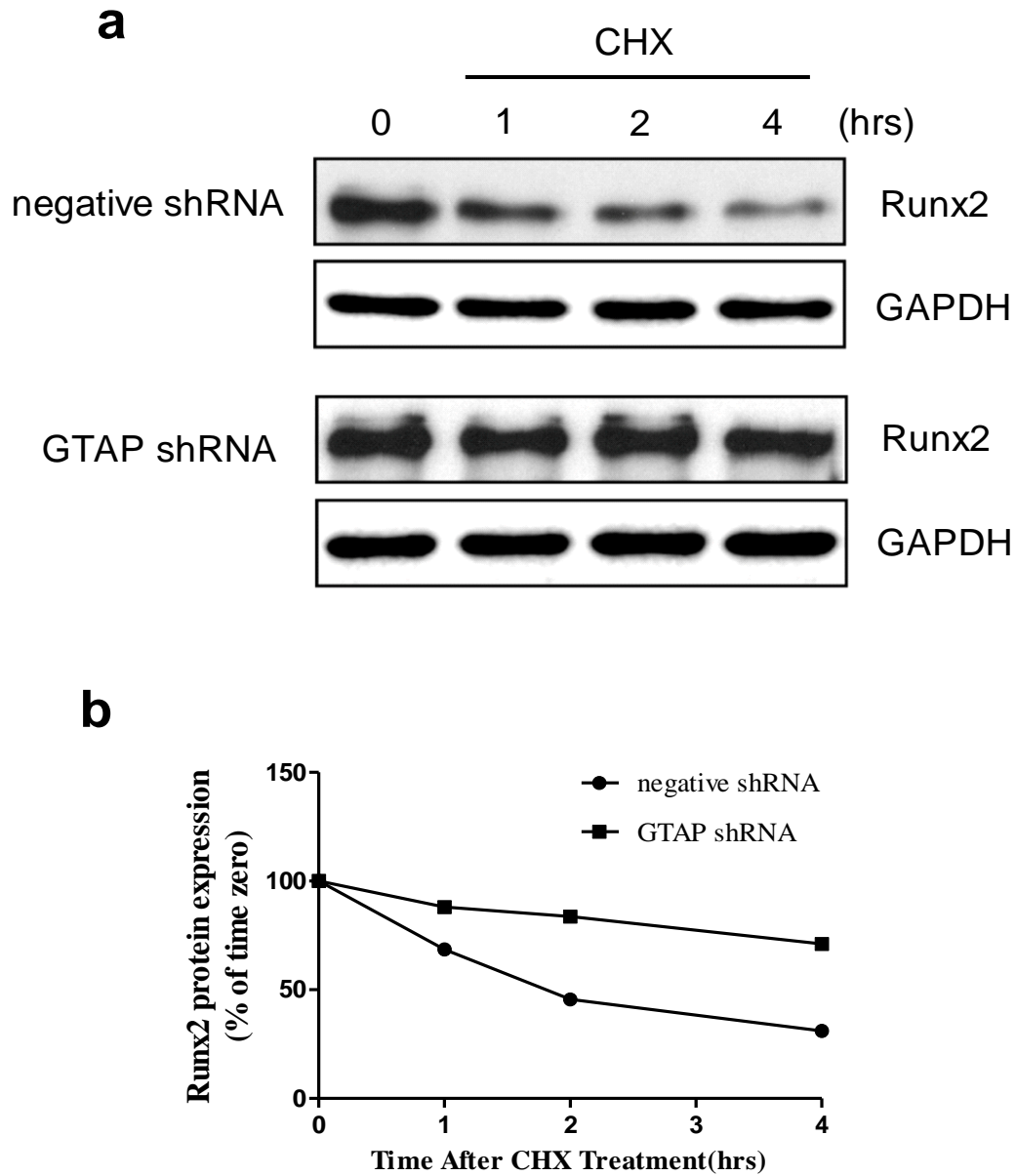


Figure 26. Western blot analysis of Runx2 level in VSMCs with decreased expression of GTAP. GTAP knockdown VSMCs were treated with cycloheximide (CHX) in culture for up to 4 hours VSMCs that express negative shRNA were used as control cells. **a.** Inhibition of protein synthesis by CHX and western blotting showing the expression of Runx2 at the indicated times (hrs). **b.** The half-life of Runx2 was estimated based on the intensity of Runx2 bands at different time points shown in **a.**

16. The requirement of the Cysteine³⁵¹ residue in the ubiquitin-binding site of GTAP for Runx2 Degradation in VSMCs

To determine whether the active cysteine residue in the ubiquitin-binding site of the C-terminus of GTAP plays a critical role in Runx2 degradation, the viral vectors with GTAP wild type or a mutant with the cysteine residue replaced by alanine at the 351 position of GTAP was delivered to VSMCs. The cells overexpressing wild type GTAP (GTAP^{WT}) or mutant GTAP (GTAP^{C351A}) were then treated with CHX. We found that Runx2 protein levels were gradually decreased in VSMCs that overexpress GTAP^{WT} in the function of CHX treatment up to 4 hours. By contrast, cells with overexpression of the mutant GTAP^{C351A} exhibited higher levels of Runx2 compared to VSMCs with GTAP^{WT} during the when treated with the same amount of CHX for the same period of time (Figure 27). This observation suggests that Runx2 degradation was compromised in VSMCs with GTAP^{C351A} mutant expression, and an active cysteine (C351) is required for Runx2 degradation.

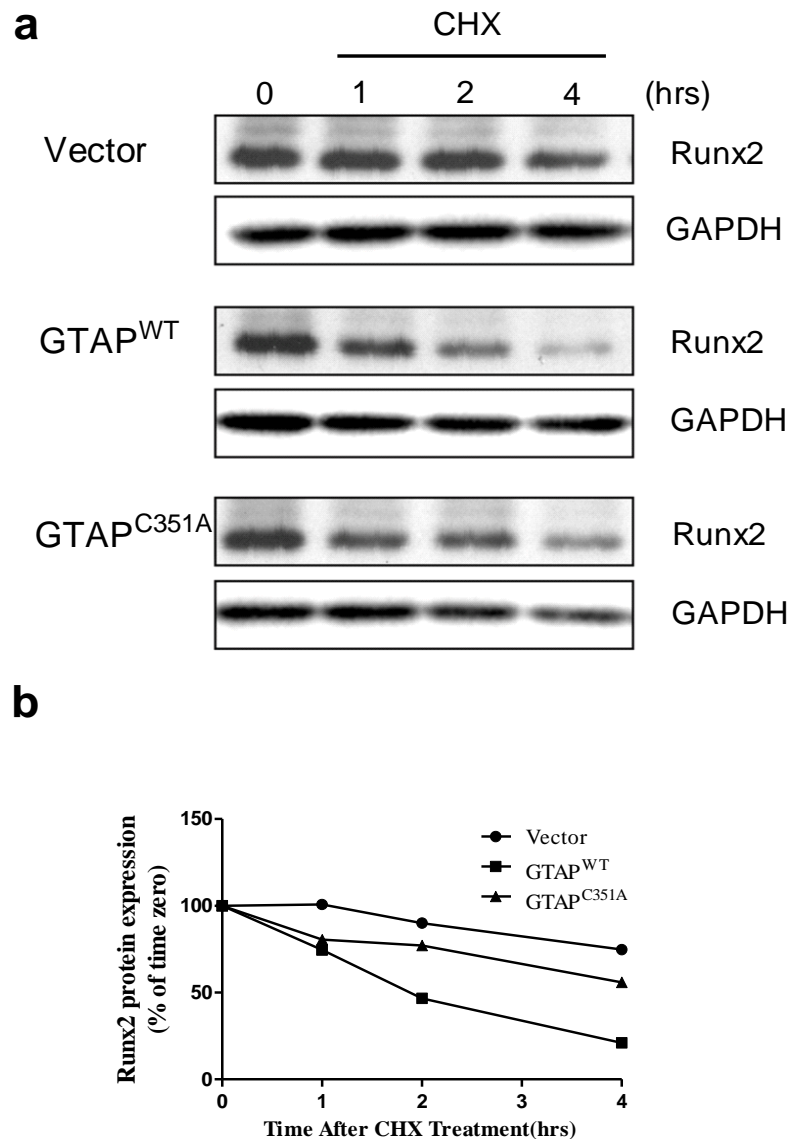


Figure 27. Western blot analysis of Runx2 level in VSMCs with GTAP overexpression. VSMCs that overpress either the wildtype form of GTAP (GTAP^{WT}) or mutant GTAP (GTAP^{C351A}) were incubated with cycloheximide (CHX) in culture for up to 4 hours. **a.** Inhibition of protein synthesis by CHX and western blotting showing the expression of Runx2 at the indicated times (hrs). **b.** The half-life of Runx2 was estimated based on the intensity of Runx2 bands at different time points shown in **a**.

Chapter 5 : Discussion

Vascular calcification represents a common biomarker of pathological changes in the arterial wall with atherosclerosis. In the first part of this study, we conducted experiments to address the issue of molecular regulation of calcification. Many reports published by other research groups have demonstrated that ApoE knockout mice develop severe atherosclerosis with mass calcification [54-56]. However, it is not clear if the vascular atherosclerotic lesions with calcification develop as a consequence of other pathobiological conditions, such as hypercholesterolemia, inflammation and/or apoptosis. Here, in the first part of this study, we show experimental evidence that ApoE serves as a key factor in regulation of VSMC calcification independently from the above risk factors. We observed that the deficiency of ApoE accelerates vascular smooth muscle cell calcification, using both *in vivo* and *in vitro* models.

Under normal chow diet, ApoE^{-/-} mice develop hypercholesterolemia and severe atherosclerotic plaques. They usually develop calcification in the regions surrounding advanced plaque tissues, often giving the impression that VC occurs secondarily to plaque generation. However, in our study, we show that ApoE^{-/-} aortic tissues with no or minor atherosclerosis can have increased calcification, indicating that calcification may be a separate or independent event from atherosclerotic lesion development. This notion is supported by our finding that VSMC cultured from ApoE^{-/-} mice in normal media with no high cholesterol contents and inflammation responses can develop severe calcification.

Aortic calcification usually occurs in mice in an age-dependent manner [92]; VC

will be accelerated under the stimuli of chemicals like vitamin D or nicotine [93, 94]. Here in our study, VSMC calcification develops in a microenvironment lacking those stimuli, but containing inorganic phosphate. Therefore, the tendency to form calcification in ApoE^{-/-} mice is likely due to the predisposed expression pattern of calcification-related genes in ApoE^{-/-} cells in the blood vessels. Apart from the susceptibility to have atherosclerotic calcification, ApoE^{-/-} mice tend to have increased bone mass and bone formation compared to wildtype ones [34]. This implies that ApoE may have an impact on osteogenic genes.

Atheroma calcification is actively regulated by a combination of inflammatory mediators, matrix components and apoptosis [4, 52, 95, 96]. Endothelial cells and monocytes are involved in the early stages of VC, while VSMCs contribute to the later stages of calcium deposition by proliferation and migration [79, 97]. Here our data showed aortic rings isolated from ApoE^{-/-} mice, when cultured in calcification-inducing medium, produced a greater extent of calcification than WT mice, suggesting factors other than inflammation and cholesterol might affect the process of calcification. Due to the complexity of the *in vivo* environment surrounding atherosclerotic calcification areas and relevant cellular players, there is not much known about whether the depletion of ApoE in one specific type of cell will alter the pattern of calcification.

In this study, VSMCs were isolated from thoracic aortas of ApoE^{-/-} and WT mice, respectively. Cells were treated with Pi and induced to calcify. We observed that ApoE^{-/-} VSMCs generated significantly more calcium mineralization than WT cells

when cultured in calcifying medium. By introducing ApoE back into ApoE^{-/-} cells, calcification was reduced dramatically. Therefore, our current study documents the first direct evidence showing that ApoE deficiency worsens calcification *in vitro*. The loss of smooth muscle lineage markers is required for the phenotypic transition from VSMCs to osteoblasts and we found that smooth muscle markers SM22 α and α SMA both have weaker expression in ApoE^{-/-} VSMCs. Osteopontin(OPN), on the other hand, is a multifunctional protein and highly expressed in bones and calcified tissues[98]. OPN promotes ectopic calcification regression, serving as an inhibitor of mineral deposition; depletion of OPN makes VSMCs more susceptible to Pi-induced calcification *in vitro* [99]. We did find that restoration of ApoE successfully rescued the decrease of SM22 α , α SMA and OPN in ApoE^{-/-} VSMCs. However, no obvious difference of Runx2 expression levels was observed between WT, ApoE^{-/-} and ApoE overexpressing cells, which indicates that smooth muscle specific markers are affected by molecular regulators other than Runx2. This is consistent with the finding that Runx2 deficiency did not alter basal SMC markers in mice [100]. Type I collagen can be produced by VSMCs and was found to be abundant in the medial layer of calcified aortic tissues and atherosclerotic plaques. *In vitro*, type I collagen matrix promotes calcification of vascular cells [101].

Apoptosis is another biological process strongly linked to vascular calcification, but the underlying mechanisms are not fully understood yet [52]. Apoptosis usually precedes VC and/or happens during the initiation of VC. It is established that apoptotic bodies and necrotic debris of VSMCs promotes nucleation of calcium and thus

enhances calcification [102]. Although it is known to protect endothelial cell against apoptosis [103], whether ApoE suppresses VC via inhibition of apoptosis in VSMCs remains unclear. However, there is evidence suggesting that ApoE^{-/-} mice are more sensitive to endoplasmic reticulum stress (ERS) [104], while ERS-related apoptosis is known to promote vascular calcification in VSMCs [105]. Therefore, it is possible that ApoE diminishes calcification by suppression of apoptosis, but the signaling pathways involved need to be identified.

The heterogeneity of VSMCs isolated from aortas has been described before [106]. A Shioi *et al.* cloned calcifying vascular cells (CVC) from bovine aortas and these cells were able to form nodules that mineralized spontaneously *in vitro* [107]. The cellular components of CVC are later identified to include VSMCs in the media, which have many features of microvascular pericytes and differ from conventional VSMCs [108]. *In vivo* studies indicated that there could be a pluripotent stem cell-like population in the artery walls, which can undergo osteogenic differentiation [109]. Hence, it is possible that ApoE^{-/-} mice aortas comprise a larger population of either CVC or osteogenesis-capable stem cells that render these mice prone to VC.

ApoE is a secreted protein and under most circumstances affects cell physiology by binding to receptors on cellular membranes. Identified ApoE receptors include apolipoprotein E receptor 2 (ApoER2), the low density lipoprotein receptor (LDLR) and the very low density lipoprotein receptor (VLDLR), most of which have been found in VSMCs [76]. Ligand-receptor interactions might be critical in protecting VSMCs against calcification. Intracellular ApoE, on the other hand, acts as a regulator of

cellular physiology as well. The accumulation of ApoE protein in endoplasmic reticulum, for instance, is closely related to the processing of amyloid precursor proteins in monkey kidney fibroblasts [110]. Even so, very few studies were done to identify the relationship between expression level and function of intracellular ApoE during calcification of VSMCs, due to the difficulty of stably expressing intracellular ApoE for the long term. Therefore, it is possible that our observation on the effect of ApoE deficiency on VC partially relies on the loss of intracellular ApoE. Further studies will be needed to better differentiate the functionality between endogenous ApoE and exogenous ApoE during calcification.

Summarizing the above discussion, when cultured under the condition in absence of hypercholesterolemia and inflammation, ApoE^{-/-} VSMCs inherently express high levels of osteogenic genes. They become more susceptible to calcification induced by osteogenic stimuli, such as inorganic phosphate, which may help explain why ApoE^{-/-} mice tend to develop vascular calcification more easily than WT mice.

Previous work by other groups have revealed different biological functions of ApoE, including regulation of glucose metabolism, lipid transport and neurogenesis[111]. However, the role of ApoE in vascular calcification and specific pathways involved has not been addressed. The data from the current study present the first experimental evidence that ApoE attenuates calcification of ApoE^{-/-} VSMCs via inhibition of the canonical Wnt/ β -catenin pathway.

Vascular calcification represents a process involving the irreversible accumulation of calcium mineralization in arteries and the subsequently hardening of

blood vessels. Hence, therapeutic strategies aimed at prevention of calcification are urgently needed. Vascular calcification shares various features with osteogenesis, both of which have ApoE as an important regulator. The best-known phenotype of ApoE^{-/-} mice is their susceptibility to develop atheroma, and calcification has been regarded as a hallmark of atherosclerosis.

Calcium deposition is commonly found in advanced atherosclerotic plaques. Senile ApoE^{-/-} mice tend to have serious valvular calcification also, especially in mice with chronic kidney failure [112]. ApoE appears to be regulated by BMP-2[33] and expressed in the later stages of osteoblast differentiation [34]. Kim *et al.* found ApoE inhibited osteoclast differentiation via c-Fos and NF-κB in bone marrow-derived macrophages [113]. Besides, a high phosphate diet and nephrectomy resulted in exacerbated vascular calcification in ApoE-deficient mice [114].

Therefore, ApoE^{-/-} mice have been used as an ideal animal model to study calcification. VSMCs, with their extensive plasticity, undergo phenotypic changes in normal development, disease states, and in response to environmental cues [50]. As the major structural component of vascular walls, VSMCs determine the stiffness of blood vessels [115]. In our study, experiments were performed in cultured VSMCs isolated from murine aortas, independent of the influence of inflammatory responses and cholesterol. Here we found ApoE rescue the loss of smooth muscle lineage marker SM22α and prevented the stimulated expressions of osteoblast markers including Runx2, ALP, BMP-2, osteopontin and osteocalcin. Further, we observed ApoE significantly reduced calcium deposition in VSMCs. This offers evidence for the

importance of ApoE in inhibiting the phenotypic transition of VSMCs into osteoblast-like cells, suggesting the possible function of ApoE in maintaining pliability of aortas.

The Wnt/ β -catenin pathway plays an indispensable part in vascular calcification. *In vitro* studies showed that the Wnt/ β -catenin pathway is activated when valvular interstitial cells differentiate into osteoblast-like cells [41]. Wnt/ β -catenin has been reported to be activated in several osteoblast or osteoblast precursor cell lines, including C3H10T1/2 cells and C2C12 cells [42], which implicate its importance in the regulation of osteogenesis. Accumulation of the pathway proteins was observed in valves, coronary arteries and aortas that undergo calcification [116, 117]. Martínez-Moreno *et al.* found that in VSMCs paricalcitol retarded phosphate-induced calcification by preventing Wnt/ β -catenin activation [118]. Beazley *et al.* noted that transglutaminase 2-mediated β -catenin activation contributed to calcification of VSMCs caused by combined treatment of warfarin and elevated concentration of phosphate [119]. When β -catenin signalling is inactive, cytoplasmic β -catenin is subject to constant degradation by Axin1 complex, which prevents it from translocating into the nucleus [120]. Binding of Wnt ligands to frizzled and LRP5/6 receptor complex recruits Axin1 to the receptor, thus enabling β -catenin to accumulate in the cytoplasm and travel to the nucleus [37]. As a response to a calcification-inducing stimulus such as high phosphorus levels, the β -catenin pathway becomes activated, and increased nuclear β -catenin acts as a transcription factor that leads to enhanced expression of downstream osteogenic genes, including osteopontin (OPN) and osteocalcin (OCN),

resulting in enhanced calcification[96]. Here we found ApoE suppressed the translocation of β -catenin into the nucleus as well as inhibited the activation of β -catenin target genes including Axin2, CyclinD1 and Tcf4 in calcifying VSMCs.

Both LRP5 and LRP6 receptors are required for canonical Wnt/ β -catenin signalling to be generated. ApoE is a ligand for LRP5 and predicted to bind to LRP6 as well. Although whether ApoE recognizes LRP5/6 as a heteromer coreceptor stays unclear, the regulatory effects of ApoE on the Wnt pathway have been observed [85, 86]. Notably, here we examined the impact of ApoE on calcification in ApoE^{-/-} VSMCs, which is depleted of intracellular ApoE. Therefore, it is very likely that the effect of ApoE we observed in our study is dependent on the interaction of ApoE and receptor(s) in VSMCs.

Together, our results suggest that ApoE attenuates vascular calcification via regulation of the canonical Wnt/ β -catenin pathway, and supplement of extracellular ApoE is sufficient to achieve substantial decrease of calcium deposition in VSMCs and significant inhibition of calcification markers, providing new potential pharmacotherapeutic options for treating vascular calcification in the future.

To date, few studies have related GTAP with calcification. In our study, we found that GTAP expression was low in aortic VSMCs derived from ApoE^{-/-} mice, which indicates ApoE might have a regulatory effect on GTAP. However, calcification induced by phosphate was dramatically alleviated in wildtype VSMCs that overexpress wildtype GTAP (GTAP^{WT}) or mutant GTAP (GTAP^{C351A}), but not in ApoE^{-/-} VSMCs with overexpression of GTAP^{WT} or GTAP^{C351A}. This implies the possibility that the

inhibition of calcification by GTAP might depend on the expression of ApoE in VSMCs; however, more results need to be obtained in order to draw a solid conclusion. In order to explore the mechanism by which GTAP alters calcification, relevant experiments were performed in our studies on mice and VSMCs that are capable of ApoE expression.

We observed that GTAP overexpression decreased the expression of Runx2, an important transcription factor necessary for osteogenesis. Moreover, enhanced expression of GTAP increased the level of smooth muscle lineage markers SM22 α and α SMA. Considering GTAP as an E2 ubiquitin-conjugating enzyme involved in protein degradation, we suspected that the low level of Runx2 caused by GTAP overexpression could result from shortened half-life of Runx2. Our data indicates Runx2 has a prolonged half-life when GTAP was knocked down. Besides, Runx2 displayed a shorter half-life in cells with wild type GTAP overexpression, while mutant GTAP which lost the ubiquitin-conjugating ability rendered Runx2 protein more stable. Hence, we might conclude that the degradation of Runx2 is partially dependent on the ubiquitin-conjugating enzyme activity of GTAP, which has never been reported before.

Grzmil P *et al.* found that GTAP-deficient (GTAP^{-/-}) female mice have embryo implantation failure and other reproduction-associated problems [87], but no obvious phenotype was detected in male GTAP^{-/-} mice. Nevertheless, we observed that aortic rings dissected from GTAP^{-/-} male mice were more sensitive to elevated concentration of inorganic phosphate (Pi) in the culture medium. Aortic rings that do not express GTAP showed a significantly higher level of calcification than wild type ones when

incubated with 3.6mM Pi. Therefore, although GTAP^{-/-} male mice don't have overt phenotypes under regular conditions, stress such as osteogenic chemicals, including Pi, might induce the calcification-prone phenotype in the absence of GTAP, suggesting GTAP possibly plays a role in protection against vascular calcification. Moreover, phenotypes of mice depend on strain. Therefore, the reason that no clear signs of vascular calcification were found in GTAP^{-/-} mice *in vivo* could be due to stronger resistance of 129/sv mice to atherosclerosis.

In this study, we found that the mutant GTAP with active cysteine replaced by an alanine alleviated calcification significantly compared to vector or wild type GTAP in VSMCs incubated with Pi. In addition, the mutant GTAP construct decreased protein expression of Runx2 moderately, though not as much as the wildtype GTAP, in VSMCs. All these results, when taken together, suggest that other than the ubiquitin-binding core (UBC) domain, the RWD domain (RING finger, WD repeats, and yeast DEAD-like helicases containing proteins) or the NH₂-terminal domain in GTAP may contribute to the regulation of calcification process as well. Of note, Alontaga AY *et al.* have studied the function of RWD domain and observed the capability of RWD domain to interact with Ubc9, another E2 ubiquitin-conjugating enzyme involved in SUMOylation, one of the modifications similar to ubiquitination [121]. Enzymatic assays revealed that the RWD domain may alleviate the inhibitory effect of Ubc9 on the catalysis of SUMO-activating enzyme. Besides, our previous findings imply that the localization of GTAP in the nucleus of embryonic stem cells (ESCs) partly depend on the NH₂-terminal domain of GTAP. The NH₂-terminal domain of GTAP is comprised of

a unique sequence which consists of multiple proline and glutamine residues often observed in small P-rich proteins, which may be involved in cell differentiation[122]. These domains are potentially important for protein transport, compartmentalization and interaction with chaperone proteins. Hence we speculate the negative regulation of calcification by GTAP probably depends on these domains as well as the UBC domain.

In conclusion, the data from the current study demonstrate: 1) Increased vascular calcification in the atherosclerotic mouse models is ApoE-dependent; 2) Vascular smooth muscle cells with ApoE knockout are sensitive to calcification induced by inorganic phosphate (Pi); 3) Pi-inducible osteogenic genes play a role in the transformation of the contractile vascular smooth muscle cells into synthetic, bone-morphogenic cells; 4) Vascular cells from atherosclerotic aortas have altered expression of the Wnt/ β -catenin pathway; and 5) GTAP can alleviate Pi-induced calcification in mouse aortas and VSMCs; 6) Potent expression of GTAP is correlated with a high expression of smooth muscle lineage markers (SM22 α and α SMA) and suppression and shorter half-life of osteogenic master regulator Runx2; and 7) GTAP may directly or indirectly regulate calcification via ubiquitylation of osteogenesis-related genes or interaction with other E2s.

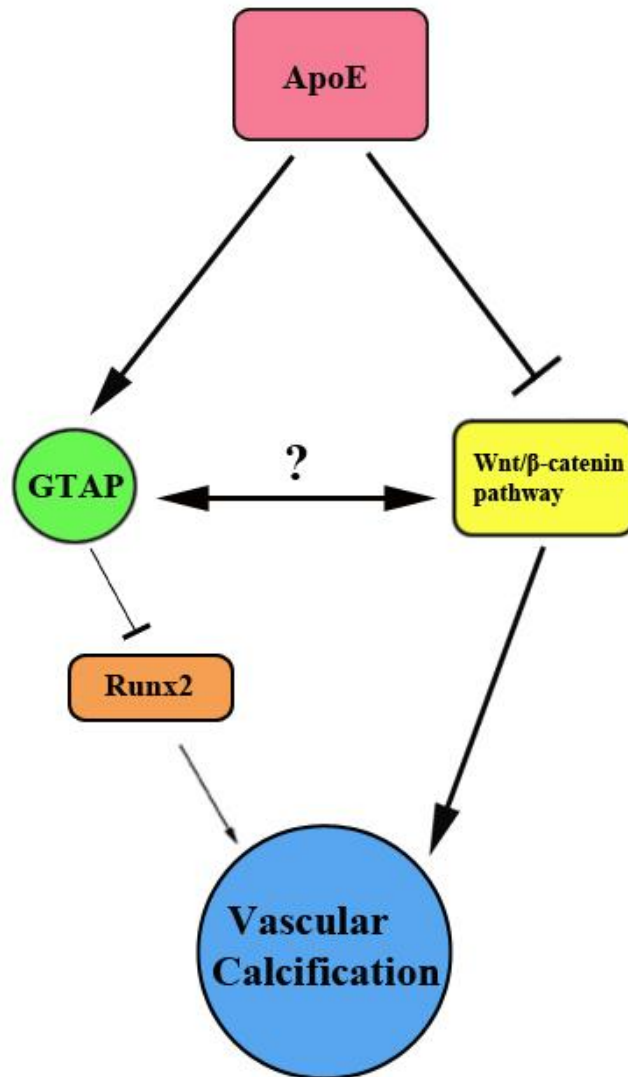


Figure 28. Proposed mechanism for regulation of calcification by ApoE in vascular smooth muscle cells (VSMCs). ApoE suppressed nuclear translocalization of β -catenin, leading to inhibition of β -catenin-dependent transcription of osteogenic genes and eventually alleviated calcification. ApoE enhanced GTAP expression, which results in a shortened half-life of Runx2, a master transcription factor involved in calcification, inhibiting the osteogenic transformation of VSMCs.

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Vita

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