The Role of Transcription Factor EB in the Vascular Wall Biology

by

Haocheng Lu

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Doctoral Committee:

Professor Y. Eugene Chen, Chair
Professor Daniel T. Eitzman
Professor Daniel E. Michele
Professor Liangyou Rui

Haocheng Lu

lhaochen@umich.edu

ORCID iD:0000-0002-5740-8010

Dedication

To my beloved wife, Wenting Hu, who supports me at all times.

To my research advisor, Dr. Yuqing Eugene Chen, who provides me with the opportunity to do research.

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Abstract

Transcription factor EB (TFEB) is a member of the microphthalmia transcription factor family, with adjacent basic helix-loop-helix and leucine zipper domains. Among them, TFEB has been found to be a master regulator of autophagy and lysosome biogenesis. TFEB is implicated in lysosomal storage diseases and neurodegenerative diseases. However, the role of TFEB in vascular biology is poorly understood. In this study, we aim to explore the role of TFEB and underlying mechanisms in vascular diseases.

Growing evidence suggests that endothelial cell dysfunction occurs in the initial stage of atherogenesis. Laminar shear stress, which protects against atherosclerosis, increased TFEB abundance in cultured primary human endothelial cells. The locations with a higher laminar shear stress of the rabbit aorta also show higher expression of TFEB. Furthermore, TFEB overexpression in endothelial cells (ECs) suppressed adhesion molecule and inflammatory cytokine expression., whereas TFEB knockdown aggravated adhesion molecule and inflammatory cytokine expression. TFEB knockdown also diminished the effect of laminar shear stress to suppress adhesion molecule and inflammatory cytokine expression in ECs, indicating TFEB to be a mediator of the anti-inflammatory and anti-atherosclerotic effects of laminar shear stress. The anti-inflammatory effect of TFEB was, at least, partially due to reduced oxidative stress because TFEB overexpression in endothelial cells decreased the concentrations of reactive oxygen species and increased the expression of the antioxidant genes HO1 (which encodes heme oxygenase 1) and SOD2 (which encodes superoxide dismutase 2). Chromatin

immunoprecipitation (ChIP) assay and luciferase reporter assay indicated that TFEB directly bound to the promoter of HO1 and SOD2. To study the EC TFEB function *in vivo*, we generated mTie2-TFEB transgenic mice, in which TFEB was overexpressed in ECs. The transgenic mice exhibited reduced leukocyte recruitment to endothelial cells and decreased atherosclerosis development in $ApoE^{-/-}$ background.

Abdominal aortic aneurysm (AAA) has a very high mortality rate in the event of rupture. It would be of high significance to identify novel strategies to prevent or treat AAA. We found that TFEB expression is reduced in the human aneurysm lesion. Both gain- and loss-of-function experiments demonstrated that TFEB inhibited the apoptosis of human aortic smooth muscle cells (HASMCs). Mechanistic studies showed that TFEB upregulated B-cell lymphoma 2 (BCL2) and BCL2 inhibitor abolishes the anti-apoptotic effect of TFEB. ChIP and luciferase reporter assays indicated that TFEB directly bound to the promoter of BCL2, suggesting BCL2 is a direct target of TFEB. To determine the role of TFEB in AAA *in vivo*, we utilized smooth muscle cell (SMC)- specific *Tfeb* knockout (KO) mice and applied two different mouse AAA models: β-aminopropionitrile/Angiotensin II- and PCSK9/Angiotensin II-induced murine aneurysm models. Consistent results were observed in the two AAA models, in which TFEB deficiency increases SMC apoptosis and promotes AAA formation. Of significance, we demonstrated that TFEB activator, 2-hydroxypropyl-β-cyclodextrin (HPβCD), attenuates aneurysm formation and inhibits HASMC apoptosis in the PCSK9/Angiotensin II model. Using SMC-TFEB KO mice, we further demonstrated that vascular smooth muscle cell (VSMC) TFEB is essential for the inhibitory effects of HPβCD on AAA formation and VSMC apoptosis *in vivo*.

Our study suggests that TFEB regulates important biological functions in the vascular wall including ECs and VSMCs. As a transcription factor, TFEB directly increases the

transcription of anti-oxidant and anti-apoptotic genes. TFEB constitutes a molecular target for the treatment or prevention of vascular diseases such as atherosclerosis and aortic aneurysms.

Chapter 1 Introduction

The RNA-sequencing result was published in the following journals:

Congzhen Qiao, Shengdi Li, Haocheng Lu, Fan Meng, Yanbo Fan, Yanhong Guo, Y. Eugene Chen & Jifeng Zhang. Laminar Flow Attenuates Macrophage Migration Inhibitory Factor Expression in Endothelial Cells. *Sci Rep.* 2018; 8: 2360.

Congzhen Qiao, Fan Meng, Inhwan Jang, Hanjoong Jo, Y. Eugene Chen, and Jifeng Zhang. Deep transcriptomic profiling reveals the similarity between endothelial cells cultured under static and oscillatory shear stress conditions. *Physiol Genomics.* 2016 Sep 1; 48(9): 660–666.

Overview

Shear stress and endothelial biology

Shear stress is the dragging frictional force of blood flow on the vascular endothelial cells. Decades of studies have shown that shear stress is critical to maintain healthy endothelial cells. Steady laminar shear stress (10-30 dyn/cm^2) keeps endothelial cells quiescent and in an anti-coagulation, anti-inflammation, pro-survival condition, while low or oscillatory shear stress induces endothelial inflammation, reactive oxygen species production and apoptosis[1]. The lesser curvature of the aortic arch and carotid blub, which experience disturbed flow *in vivo*, are more susceptible to develop atherosclerosis[1] and this is speculated to be due to the disrupted laminar

sheer stress in these regions compared to the laminar flow observed in other parts of the vasculature. Direct experimental evidence also comes from experiments using a perivascular carotid artery cuff to disturb local shear stress in an otherwise laminar vessel. These experiments show that local shear stress pattern contributes directly to the susceptibility to atherosclerotic plaque formation in mice[2].

Several mechanosensors in the endothelial cells have been found and transduce the extracellular mechanical force to intracellular signal pathways, including Occludin, mechanosensory complex, focal adhesion molecules, and ion channels[3]. The phenotypic changes induced by shear stress are mediated by several pathways, including nitric oxide[4], mitogen-activated protein kinase (MAPK)[5], and protein kinase C (PKC)[6] pathway in endothelial cells. These pathways merge at several transcription factors to influence the gene transcription under shear stress treatment. The first transcription factor identified to be uniquely induced by flow in endothelial cells is Krüppel-like factor 2 (KLF2)[7]. The target genes of KLF2 in ECs include adhesion molecules, cytokines[8], and metabolic genes[9]. Another shear stress-responsive transcription factor is nuclear factor (erythroid-derived 2)-like 2 (Nrf2), which coordinates the expression of anti-oxidative genes in the ECs[10]. The elucidation of these shear stress-regulated pathways increases our knowledge of endothelial cell biology and have potential to become novel therapeutic targets.

Deep transcriptomic profiling of endothelial cells cultured under different shear stress conditions

To understand the pathways mediating the protective effect of laminar shear stress, we utilized RNA-sequencing technology to profile the transcriptome of ECs under steady laminar shear stress (LS) (15 dyn/cm^2), oscillatory shear stress (OS) (0 ~ ±5 dyn/cm^2), and static

condition (ST)[11, 12]. We found that more than 8000 genes were differentially expressed between LS vs OS or LS vs ST, while only 1618 genes were differentially expressed between OS and ST, which indicates that EC under laminar shear stress display a distinct transcriptome.

TFEB is upregulated by laminar shear stress in deep transcriptomic profiling

Among the differentially expressed genes, we found that transcription factor EB (*TFEB*) is significantly upregulated by LS, compared with OS or ST (Fig. 1.1). TFEB has emerged as a master regulator of lysosomal biogenesis[13]. However, the role of TFEB in vascular biology is poorly understood. Since TFEB can be upregulated by laminar shear stress in ECs, we hypothesized that TFEB may be a regulator of the vascular biology and partially mediate the protective effect of laminar shear stress on endothelial biology. In this dissertation, I will focus on the role of TFEB in vascular biology.

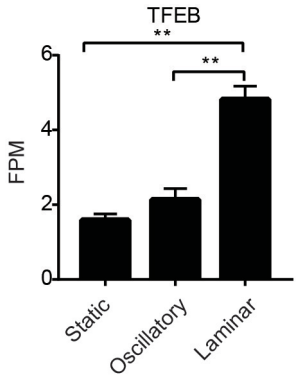

Figure 1.1 TFEB is upregulated by laminar shear stress.

Transcriptome of ECs under static condition, oscillatory shear stress, and steady laminar shear stress was profiled by RNA-sequencing. TFEB mRNA abundance was shown as fragments per million mapped fragments (FPM). Data were presented as mean ± SEM. N=4 for each group. **p<0.01.

TFEB Biology

MiTF/TFE gene family

TFEB is a member of the microphthalmia transcription factor family, together with microphthalmia transcription factor (MiTF), transcription factor EC(TFEC), and transcription factor E3(TFE3)[14]. They contain similar adjacent basic helix-loop-helix and leucine zipper domains (bHLH-Zip) and bind to E-box (CANNTG) as either homodimers or heterodimers to regulate target gene transcription[15]. *TFE3* was cloned with a fragment of the μE3 motif within the immunoglobin heavy-chain enhancer[16]. *TFEB* was screened from a cDNA library from a human B-cell line, with the ability to bind to a sequence in the major late promoter of adenovirus[17]. *MiTF* was first cloned from a locus, in which mutation causes microphthalmia phenotype in mice. *TFEC* was cloned from a rat chondrosarcoma tumor cDNA library based on its similarity with *TFE3*[18]. Disruption of *Mitf* by transgene insertion leads to the defection of pigmentation, eye size, bone development, mast cell, and hearing in mice[19]. *Tfec* or *Tfe3* knockout mice are phenotypically normal and further study has shown that *Mitf* and *Tfe3* have redundant function in osteoclast development[20].

TFEB global KO is embryonic lethal.

To elucidate the biological function of TFEB, the global *Tfeb* knockout mice were generated by homologous recombination in embryonic cells[21]. Distinct from other family members, *Tfeb* deficient mice die between 9.5-10.5 days in the uterus due to the impaired placental vascularization[21], underscoring the importance of TFEB in embryo development.

TFEB involved in renal cancer

The fusion of *TFEB* gene and *Alpha* gene by chromosome translocation t(6;11)(p21.1;q12) has been reported to be involved in the renal tumor. The fusion happens at the 5' untranslated region of *TFEB*, leaving the coding sequence of *TFEB* intact in the genome, but promotes the abnormal expression of *TFEB*[22, 23]. Other members in *MiTF/TFE* gene family, such as *MiTF* and *TFE3*, have also been reported to fuse with other genes in the human tumor samples, indicating a common role of MiTF/TFE family proteins in tumorigenesis[24].

TFEB is a master regulator of lysosomal biogenesis and autophagy.

In 2009, TFEB was found to be a master regulator of lysosomal biogenesis and regulates a variety of lysosomal genes by binding to the palindromic 10–base pair (bp) GTCACGTGAC motif. This motif is named Coordinated Lysosomal Expression and Regulation (CLEAR) element, because of its enrichment in the promoter of lysosomal genes. Importantly, only TFEB, but not other members in the MiTF/TFE family, can activate the transcription of lysosomal genes[25]. Autophagy is an important process for the cell to clean and recycle damaged molecules and organelles. Autophagosome requires to be fused with lysosomes to degrade the protein and other macro molecules[26]. Consistent with this, TFEB also upregulates autophagy genes, together with lysosomal genes, to promote the autophagy, which is an important adaption for cells under starvation and stressed conditions[13].

Regulation of TFEB

Regulation of TFEB by post-translational modification

TFEB activation is mainly regulated by phosphorylation. Numerous phosphorylation site of TFEB has been identified. (1) The mechanistic target of rapamycin (mTOR) dependent

phosphorylation of Ser211 promotes TFEB binding to 14-3-3 protein and inhibits its nuclear translocation[27, 28]. (2) In osteoclasts, Protein Kinase Cβ (PKCβ) phosphorylates TFEB at Ser461/462, Ser466, and Ser468, which is required for RANKL induced TFEB activation. (3) The mechanistic target of rapamycin complex 1 (mTORC1)[29] and extracellular signal-regulated kinases (ERK)[30] phosphorylate Ser142 of TFEB and retains TFEB in the cytoplasm. (4) A recent discovery found that Ser122 is also phosphorylated by mTORC1 and the dephosphorylation of Ser122 is essential for TFEB nuclear localization[31]. (5) TFEB Ser142 phosphorylation primers the Ser138 phosphorylation by GSK3β and both phosphorylation activate nuclear export signal for TFEB shuttling between nuclear and cytoplasm[32]. (6) STIP1 homology and U-Box containing protein 1 (STUB1) preferentially target inactive phosphorylated TFEB to proteasome degradation[33]. (7) On the other hand, calcineurin, a phosphatase activated by intracellular Ca^{2+}, dephosphorylates TFEB and induce its nuclear translocation[34]. (8) Protein phosphatase 2A also dephosphorylates TFEB at Ser211 and stimulates its nuclear translocation[35].

Besides phosphorylation, acetylation of lysine residual (K91, K103, K116, and K430) has been reported to promote TFEB activity[36].

The elucidation of these post-translational modifications of TFEB provides the pharmaceutical target to manipulate TFEB activity in the cells.

Regulation of TFEB by transcription.

TFEB is also regulated at the transcription level by several transcription factors. TFEB is induced in mice liver by starvation via a positive autoregulatory loop by binding to its own promoter region and promoting its own transcription[37]. (2) In Huntington's disease mice, TFEB mRNA expression is compromised and peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) can bind to TFEB promoter to active its transcription and attenuate the

disease phenotype[38]. (3) In the mouse liver, cAMP response element-binding protein (CREB), together with its co-activator CREB regulated transcription coactivator 2 (CRTC2), up-regulates TFEB transcription. whereas farnesoid X receptor (FXR) disrupts this complex and inhibits TFEB transcription[39]. (4) In mouse astrocytes, PPARα-RXRα-PGC1α complex is capable of up-regulating TFEB transcription via PPAR response element in the promoter[40]. (5) In adipocytes, FOXO1 directly increases TFEB transcription [41]. (6) Kruppel-like factor 2 (KLF2) increases TFEB transcription in EC under laminar shear stress[42]. These studies indicate a cell-dependent transcriptional regulation of TFEB and may provide the molecular basis of TFEB to be involved in various diseases.

TFEB in cardiovascular and metabolic diseases.

TFEB in endothelial biology

The study of TFEB in endothelial biology is very limited. Our lab previously found that TFEB promotes postischemic angiogenesis via activating AMP-activated protein kinase α (AMPKα) signaling and autophagy pathway[43]. In human brain microvascular endothelial cells, TFEB-mediated lysosomal protein degradation pathway inhibits TNFα induced ICAM1 expression[44].

TFEB in macrophages

TFEB is essential for innate immune response and macrophage activation against microbial infections[45-48]. TFEB downregulation facilitates the alternative activation of macrophage in the tumor microenvironment and enhances tumor growth[49]. In the mouse atherosclerosis model, accumulation of excessive intracellular cholesterol impairs normal lysosome function in the macrophage. TFEB overexpression in macrophages rescues normal

lysosome function and reduces inflammasome activation and IL1β production. Intriguingly, the inhibition of IL1β is autophagy independent as *Atg5* knockout (KO) did not abolish this effect[50]. Macrophage-specific TFEB overexpression (TFEB transgene) *in vivo* promotes lysosomal biogenesis and autophagy in atherosclerotic plaque and reduces lesion size in an Atg5 and p62 dependent manner[51].

TFEB in cardiomyocytes

In the cardiovascular system, growing evidence shows that TFEB is an important transcription factor, regulating cell survival in cardiomyocytes under various conditions in the heart.

In cardiac ischemia-reperfusion injury, Reactive oxygen species (ROS) generation and hypoxia-inducible protein BCL2 interacting protein 3 (BNIP3) upregulates beclin-1 and therefore inhibits TFEB transcription, indicating negative feedback between beclin-1 and TFEB. TFEB activation, together with PGC1α, restores mitochondria biogenesis and suppress cardiomyocytes death[52]. Repetitive intermittent fasting protects against myocardial ischemia-reperfusion in mice and endogenous TFEB is required for this attenuation of hypoxia-reoxygenation-induced cell death[53]. Senescence suppresses TFEB nuclear translocation and autophagy in the mouse heart, resulting in increased susceptibility of LPS-induced myocardial injury in aged mice[54]. Conditional KO of *Rag GTPases A* and *B (RagA* and *RagB*), which are involved in TFEB phosphorylation by mTOR, in skeletal and cardiac muscle tissues results in hypertrophic cardiomyopathy and phenocopies lysosomal storage diseases. This effect cannot be rescued by constitutively activated TFEB, marking the complexity of lysosome and autophagy regulation in cardiomyocytes[55]. TFEB overexpression could also attenuate cardiac proteotoxicity in mice with cardiac-specific expression of a missense (R120G) mutant αB-crystallin[56].

TFEB inhibition has been reported to be related to the cardiac toxicity of certain metabolites or drugs. Glucolipotoxicity suppress TFEB, leading to diminished lysosome function in cardiomyocytes[57]. Oxidative Stress induced by Monoamine Oxidase-A (MAO) leads to cardiomyocytes necrosis and heart failure via impairing TFEB activation. MAO activation promotes reactive oxidative species (ROS) production, blocks TFEB nuclear translocation and autophagic flux, while TFEB overexpression ameliorates autophagosome accumulation and mitochondria fission[58]. In addition, doxorubicin (DOX), a cancer chemotherapy drug, cause cardiomyopathy as a rare but severe side effect[59]. Bartlett et al., discovers that TFEB suppression by DOX mediates this cardiac toxicity. Restoration of TFEB prevents ROS production, caspase activation, and cell death[60]. Furthermore, cardiac stem cells cultured from explanted failing hearts are characterized by defective TFEB activation and autophagy, compared with cardiac stem cells from healthy donors, while mTOR inhibition activated TFEB and restores the autophagy function in senescent cardiac stem cells[61].

TFEB in metabolic diseases

Metabolic diseases, including obesity, dyslipidemia, and diabetes are risk factors for cardiovascular diseases. Recent evidence reveals the indispensable role TFEB in regulating whole-body metabolism and energy expenditure.

TFEB, together with TFE3, directly binds to peroxisome proliferator-activated receptor γ2 (Pparγ2) promoter in adipocytes and mediates the effect of adiponectin on blood glucose in mice[62]. TFEB is also essential for the metabolic adaptation of skeletal muscle during exercise. TFEB promotes glucose uptake, glucose utilization, mitochondria biogenesis, fatty acid oxidation, and ATP generation during exercise[63]. TFEB in hepatocytes controls lipid catabolism via inducing PGC1α transcription. TFEB overexpression by helper-dependent adenovirus

injection reduces body weight and rescues metabolic syndrome in high-fat diet fed mice[64]. *Tfe3* KO mice show more severe obesity and diabetes after high-fat diet and TFEB overexpression can compensate for metabolic abnormality caused by TEF3 deficiency[65]. In adipocyte, FOXO1 increases mitochondrial uncoupling proteins (UCP1, UCP2, and UCP3) via up-regulation of TFEB transcription[41].

As a result, activating TFEB and autophagy has emerged as a promising strategy for cardiovascular and metabolic diseases[66].

TFEB as a potential drug target

As a master regulator of lysosomal function, TFEB becomes an attractive target for lysosomal storage disorders (LSDs). LSDs are a group of diseases caused by a lack of important enzymes in lysosomal degradation pathways, which subsequently leads to the accumulation of a certain substance (glycosaminoglycans, sphingolipids, glycogen, and proteins) in the cellular compartments. Activation of TFEB can enhance the ability of cells to clear these substrates by enhancing lysosomal biogenesis and function[67]. Another interesting field regarding the implication of TFEB is the neurodegenerative diseases, which are characterized by intracellular aggregates in the brain[68, 69]. TFEB activation or overexpression successfully attenuates various neurodegenerative diseases in mouse models, including Alzheimer's disease[70, 71], Parkinson's disease[72, 73] and Huntington's disease[74], and thus is promising for treating human neurodegenerative diseases.

The nuclear translocation of transcription factor EB is under strict control. As a result, most drugs targeting TFEB influence its intracellular location and activity. There have been a number of compounds that can activate TFEB in different tissue and cell types through different mechanisms in publications. (1) An analog of curcumin, termed C1, was identified to directly

bind to TFEB and facilitate its nuclear translocation via inhibiting mTOR. Administration of this analog promotes autophagy both *in vitro* and in rat brain[75]. (2) 2-Hydroxypropyl-β-cyclodextrin (HPβCD), an excipient in drugs, has been revealed as a TFEB activator and enhances the cellular autophagic clearance of intracellular proteolipid aggregates[76]. (3) Trehalose (α-D-glucopyranosyl α-D-glucopyranoside), increases TFEB nuclear translocation by inhibiting Akt mediated TFEB Ser467 phosphorylation. Trehalose promotes the clearance of proteolipid and prolongs the lifespan in Batten disease mouse model (a neuron LSD)[77]. Besides LSDs, trehalose is also effective in macrophages to induced TFEB activity and reduces atherosclerotic lesion size in $ApoE^{-/-}$ mice[78]. (4) HEP14 (5β-O-angelate-20-deoxyingenol) and HEP15 (3β-O-angelate-20-deoxyingenol), compounds from plants, activate TFEB and autophagy in a PKC dependent pathway. Administration of HEP14 to an Alzheimer's disease mouse model ameliorate the amyloid β plaque formation[79]. (5) Ezetimibe, a prescribed cholesterol-lowering drug, activates TFEB nuclear translocation in a MAPK/ERK-dependent pathway. Ezetimibe promotes autophagy flux and attenuates lipid accumulation and inflammation in nonalcoholic steatohepatitis mice liver. This effect is abolished in atg7 KO mice, showing an indispensable role of autophagy[80]. (6) A nanotechnology-enabled high-throughput screen identified three novel compounds (digoxin, alexidine, and ikarugamycin) as agonists of TFEB and autophagosomal activity. These molecules activated TFEB via different Ca^{2+}-dependent pathways[81]. An oral supplement of digoxin or intravenous injection of alexidine or ikarugamycin attenuated the metabolic syndrome in mice. Other TFEB activators and their mechanisms to activate TFEB are listed in Table 1.1.

Name	Mechanism	*In vitro* study	*In vivo* study	Ref

Gemfibrozil	PPARα-RXRα-PGC1α	Astrocytes: Increase TFEB expression and lysosomal biogenesis	Increase TFEB expression and lysosomal biogenesis in the cortex	[40]
Gypenoside XVII		PC12: Elimination of AβPP, Aβ40, and Aβ42 protein	APP695 (APP695swe) and APP/PS1 mice: prevented the formation of Aβ plaques in the hippocampus and cortex of APP/PS1 mice	[82]
GDC-0941		Increase lysosomal function in glioblastoma cells		[83]
Docetaxel	Reactive oxygen species	Different cancer cells	ROS (reactive oxygen species) generation	[84]
Naringenin	Reactive oxygen species	Peritoneal macrophage: Inhibits cytokine production	Attenuate LPS-induced endotoxemia and acute liver inflammation	[85]
Carbon monoxide	PERK-calcineurin	Hepatocyte: Increase mitophagy	Reduces liver injury in LPS/D-GalN injected mice.	[86]

Table 1.1 List of other TFEB activators. The table summarizes the TFEB activators and their mechanism to activate TFEB.

Summary

Accumulating evidence indicating TFEB as an important regulator of lysosomal biogenesis and other intracellular processes, including inflammation and cell survival. These results enrich our understanding of the target genes of this transcription factor and the signaling pathway it is involved. The understanding of the dynamic regulation of TFEB subcellular location enables us to develop compounds to manipulate its activity. Currently, most of the studies regarding TFEB biology is related to lysosomal storage disease and neurodegenerative disease. To understand the biology of TFEB in vascular wall biology and cardiovascular diseases would help to understand the pathophysiology of these diseases and provide us novel therapeutic targets. From the preliminary date, TFEB is upregulated by protective laminar shear stress. We hypothesize that TFEB is an important regulator of vascular biology. In chapter 2, we investigated the role of TFEB in endothelial inflammation and atherosclerosis. In chapter 3, we studied the role of TFEB in the vascular smooth muscle cell apoptosis and aortic aneurysm. In chapter 4, we discussed TFEB to be a potential pharmaceutical target for cardiovascular diseases.

Chapter 2 TFEB in endothelial inflammation and atherosclerosis

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Introduction

Every year, over 30% of all death in the US is attributable to cardiovascular diseases (CVDs), such as myocardial infarction, stroke or ischemic heart failure (WHO, 2014) and total direct and indirect cost of CVDs and stroke in the United States for 2010 was estimated to be over $315 billion[87]. Atherosclerosis causes most of the pathogenesis in CVDs[88]. Atherosclerosis is a progressive disorder of the vascular wall characterized by abnormal accumulation of lipid and immune cells in the subendothelial region. Growing evidence suggests that endothelial cell dysfunction occurs in the initial stage of atherogenesis and contributes to the formation, progression, and complication of the atherosclerotic plaque[89]. Atherosclerosis is considered to be

an inflammatory disease[90, 91] and inflammatory responses are an important hallmark of endothelial dysfunction[92]. Many pro-inflammatory factors such as oxidized low-density lipoprotein (oxLDL), tumor necrosis factor α (TNFα), interleukin-1 (IL1) and other activate endothelial cells and lead to recruitment of circulatory monocytes and leukocytes.

Accumulating evidence reveals a causal relation between oxidative stress and endothelial inflammation [93, 94]. In the cardiovascular system, reactive oxygen species (ROS) exert critical physiological roles in controlling endothelial cell function and vascular tone and pathophysiological roles in inflammation, hypertrophy, proliferation, apoptosis, migration, fibrosis, angiogenesis, vascular remodeling, etc.[95-98]. Excessive ROS lead to inflammation and endothelial dysfunction *in vitro* and *in vivo* [95, 99]. Endogenous ROS are involved in the pathophysiology of atherosclerosis by increasing the abundance of adherent molecules such as E-selectin, intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1) and chemotactic factors such as Interleukin 6 (IL6), monocyte chemoattractant protein1 (MCP1)[94], leading to recruitment of inflammatory cells.

TFEB is a basic helix-loop-helix transcription factor and an autophagy master gene, regulating lysosomal biogenesis and autophagy function in many cell types[100]. In macrophages, TFEB induces lysosomal biogenesis and rescues lipid-induced lysosomal dysfunction in atherosclerotic lesion[101]. In the heart, oxidative stress induced by monoamine oxidase-A impairs the transcriptional activity of TFEB[102] and TFEB deficiency suppresses autophagy [103] and leads to cell death[104].

In the present study, we report that TFEB abundance was increased by laminar shear stress. Overexpression of TFEB in endothelial cells potently inhibited inflammation, while knockdown of TFEB aggravated inflammation. TFEB reduced intracellular ROS by increasing

the abundance of anti-oxidant genes such as heme oxygenase 1 (*HO1*) and superoxide dismutase 2 (*SOD2*). Mice overexpressing TFEB in an endothelial cell-specific manner exhibited decreased EC-leukocyte adhesion under inflammatory conditions *in vivo*. In addition, *EC-TFEB* transgene inhibits atherosclerotic lesion formation in *APOE*$^{-/-}$ mice.

Materials and methods

Animal procedures

EC-specific TFEB transgenic mice (*EC-TFEB*) were generated with a mTie2 promoter-driven human TFEB coding region on C57BL/6 mice background. Mice had free access to water and rodent chow diet. *EC-TFEB*/*ApoE*$^{-/-}$ mice were generated by breeding *EC-TFEB* mice with *ApoE*$^{-/-}$ mice (Jackson Laboratory, Bar Harbor, ME). Eight- to 10-week-old *EC-TFEB*/*ApoE*$^{-/-}$ mice and littermate *ApoE*$^{-/-}$ mice were fed high-cholesterol diet (HCD, 17.3% protein, 21.2% fat, 48.5%carbohydrate, 0.2%cholesterol by mass, and 42% calories from fat; TD.88137, Envigo, Indianapolis, IN) for 8 weeks. Blood samples were sent to the Chemistry Laboratory of the Michigan Diabetes Research and Training Center to determine total cholesterol (TC), triglycerides (TG), LDL-c and HDL-c. All animal work was performed in accordance with the University of Michigan Animal Care and Use Committee.

Materials and reagents

Antibodies against E-selectin, VCAM1, GAPDH, p65, and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against p-4EBP1, 4EBP1, TFEB, histone 3 were from Cell Signaling Technology (CST, Danvers, MA). Flag antibody was Sigma-Aldrich (St. Louis, MO). Recombinant human TNFα and IL1β were from R&D systems (Minneapolis, MN). Chloroquine, 3M-A, and LPS were from Sigma-Aldrich (St. Louis, MO). Bafilomycin A1 was from Cayman Chemical (Ann Arbor, MI).

Cell culture and stimulation

Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) were purchased from Lonza. HUVECs were cultured in M199 medium supplemented with 16% fetal bovine serum (FBS), 1ng/ml of recombinant human fibroblast growth factor (Sigma, St. Louis, MO), 90μg/ml of heparin and 20mM HEPES and 50mg/ml of Pen/Strep mix at 37°C/5% CO2 humidified incubator. HCAEC was cultured in EGM™-2MV medium (Lonza, Basel, Switzerland). Endothelial cells of passage less than 5 were used for experiments. Bovine aortic endothelial cells (BAEC) and AD-293 cells (ATCC, Manassas, VA) were cultured in DMEM with 10% FBS. Thioglycollate-elicited peritoneal macrophages and bone marrow monocytes were isolated from both *wild-type* and *EC-TFEB* mice as described previously [105, 106]. The purity of isolated cells was measured by sorting with F4/80 antibody. Endothelial cells were stimulated with TNFα (2ng/ml), IL1β (5ng/ml), or LPS (100 ng/ml) for 4 hours, unless otherwise indicated. To block autophagy, endothelial cells were pretreated with 3-MA (5mM), bafilomycin A1 (200nM), or chloroquine (5μM) for 30 min or 16 hours, and then treated with TNFα (2ng/ml) for 4 hours.

Shear stress model

A detailed protocol has been described previously [107]. Briefly, HUVECs monolayers at 80-90% confluence in 100-mm tissue culture dishes were exposed to arterial levels of unidirectional laminar shear stress (15 dyn/cm2), bidirectional oscillatory shear stress at 1 Hz cycle (±5 dyn/cm2) by rotating a Teflon cone (0.5° cone angle) with a stepping motor (Servo Motor) and computer program control (DC Motor Company, GA), and static cultured condition for the indicated time (n=3, respectively). siRNA was administrated 48 hours prior to shear stress treatment, followed by 24-hour shear stress treatment.

2', 7'-Dichlorodihydrofluorescein Diacetate (DCFH-DA) Assay

DCFH-DA assay was performed with Cellular ROS/Superoxide Detection Assay Kit (Abcam, UK) in accordance with the manufacturer's protocol. In brief, HUVECs were washed with PBS and treated with 20μM DCFH-DA and TNFα at indicated doses and time. The plate was read with 488/520 fluorescence filter by a fluorometer (Promega, Madison, WI) or visualized with fluorescence microscopy. The fluorescence was normalized to protein content in each well.

DHE superoxide assay

Superoxide production was assessed by DHE (Cayman Chemical, Ann Arbor, MI) fluorometric assays. In brief, HUVECs were washed with PBS and treated with 5μM DHE and TNFα 10ng/ml for 1 hour. The plate was read with 510/595 fluorescence filter by a fluorometer (Promega, Madison, WI). The fluorescence was normalized to protein content in each well.

Luminol chemiluminescence assay

Luminol Chemiluminescence assay was performed as described before [108]. In brief, HUEVCs were treated with TNFα at indicated dosage and time. After washing with PBS twice, cells were loaded with 1mM luminol (Cayman Chemical, Ann Arbor, MI). Luminescence was detected with Luminometer (Promega, Madison, WI) and normalized to protein content as determined by Bradford assay.

Intravital microscopy

Eight- to 10-week old *EC-TFEB* mice and *wild-type* mice were administered saline or Chloroquine (50mg/Kg, i.p.) for 1 week. Intravital microscopy analysis was performed as described before [109]. In brief, mice were injected LPS (30μg/kg) by tail vein. Four hours later, mice were injected Rhodamine 6G Chloride (Thermo Scientific, Waltham, MA) to stain

leukocytes for 20 min. The cremaster muscle was dissected from surrounding tissues, cut longitudinally and kept flat by silk suture. The muscle was kept moist by saline at 37 °C. Intravital microscopy was used to monitor microcirculation. Leukocyte rolling was quantified by counting the number of cells rolling pass a fixed point in a minute. Leukocytes that stay stationary for more than 30s were counted as adherent to EC. All animal work was performed in accordance with the University of Michigan Animal Care and Use Committee.

Immunostaining

EC-TFEB and littermate *wild-type* mice were anesthetized with ketamine (50mg/kg) and xylazine (5mg/kg). The thoracic aorta was harvested and then fixed in 4% paraformaldehyde. The sample was embedded in O.C.T (Thermo Scientific, Waltham, MA) and cut to the 8μm section in a Leica cryostat. The section was blocked in 5% goat serum for 1 hour at room temperature and then incubated with primary TFEB antibody (Bethyl laboratories, A303-673A, TX, USA), VCAM1(Abcam, UK), CD31 (HistoBioTec LLC, FL, USA) 1:100 at 4 °C overnight. After washing with PBS, the sample was incubated with Alexa fluor-labeled secondary antibody (Jackson ImmunoResearch laboratory, PA, USA) 1:1000 at room temperature for 1 hour. Images were obtained with an Olympus IX73 microscope. Background correction was performed using the appropriate IgG negative controls. For en face immunostaining of TFEB on the rabbit aorta, the different regions of the aorta were harvested from wild-type New Zealand white rabbits and fixed in 4% paraformaldehyde. The samples were incubated with primary TFEB antibody (Biorbyt, orb332323, CA, USA) 1:100 for 2 days. After washing with PBS, the sample was incubated with Alexa fluor-labeled secondary antibody (Jackson ImmunoResearch laboratory, PA, USA) 1:1000 at room temperature for 1 hour. Images were obtained with a Nikon A1

confocal microscope. Background correction was performed using the appropriate IgG negative controls.

RNA preparation and RT-qPCR analysis

Total RNA was extracted from cells using RNeasy Kit (QIAGEN, Hilden, Germany), followed by reverse transcription with SuperScript III kit (Invitrogen, Carlsbad, CA) and random primers. mRNA was determined by qPCR (BioRad, Hercules, CA), using iQ SYBR Green Supermix (BioRad, Hercules, CA). The mRNA level was normalized to internal control, GAPDH unless otherwise mentioned. The primers used are shown in Table 2.1.

Gene*	Primer sequence
hTFEB	Forward: gcggcagaagaaagacaatc
	Reverse: ctgcatcctccggatgtaat
hVCAM-1	Forward: cgaacccaaacaaaggcaga
	Reverse: acaggattttcggagcagga
hE-selectin	Forward: actttctgctgctggactct
	Reverse: tagttccccagatgcacctg
hMCP1	Forward: ccccagtcacctgctgttat
	Reverse: tggaatcctgaacccacttc
hIL6	Forward: atgcaataaccacccctgac
	Reverse: atctgaggtgcccatgctac
hHO1	Forward: attctcttggctggcttcct
	Reverse: cccctctgaagtttaggcca
hSOD2	Forward: agggaaacactcggctttct
	Reverse: ttgcctttactgtgcaggtg
HO1 ChIP	Forward: tatgactgctcctctccacc
	Reverse: ctgaggacgctcgagagg
SOD2 ChIP	Forward: cctggtgttccccttatct
	Reverse: tccttcaccgaaaactccag
hGAPDH	Forward: ccaaggagtaagacccctgg
	Reverse: tggttgagcacagggtactt

18S RNA	Forward: catggccgttcttagttggt
	Reverse: cgctgagccagtcagtgtag
hATG3	Forward: ctggcggtgaagatgctatt
	Reverse: gtggcagatgagggtgattt
hATG9B	Forward: agggtttcaggtgaccacag
	Reverse: cacttgaccctgcactctga
hSQSTM1	Forward: ctgcctcctggtctcttcac
	Reverse: gttaggagggacagggttcc
hLAMP1	Forward: ctgcctttaaagctgccaac
	Reverse: tgttctcgtccagcagacac
hATP6V1H	Forward: tctggaaggttggaatggag
	Reverse: ttctccaacatcgtgagcag
hCTSd1	Forward: gacacaggcacttccctcat
	Reverse: ctctggggacagcttgtagc
hHEXA	Forward: gcacctttggaccagtgaat
	Reverse: cttgaagtcctcaccgaagc
hGNS	Forward: gtttaagggacccactgcaa
	Reverse: ctttgcatgagagggagagc
hGLA	Forward: agcctgggctgtagctatga
	Reverse: tgcctgtgggatttatgtga
mVcam1	Forward: acagacagtcccctcaatgg
	Reverse: acagtgacaggtctcccatg
mE-selectin	Forward: gtctagcgcctggatgaaag

	Reverse: tgaattgccaccagatgtgt
mTie2- TFEB genotyping	Forward: gcggcagaagaaagacaatc
	Reverse: cattgatgagtttggacaaaccac

Table 2.1 Primers used for Real-time PCR and genotyping. h=human m=mouse

Protein extraction and Western blot

Cells were lysed in RIPA lysis buffer (Thermo Scientific, Waltham, MA) with a protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Proteins were resolved in 10% SDS-PAGE gel and transferred to nitrocellulose membrane (BioRad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in TBST containing 5% fat-free milk and incubated with primary antibody (1:1000) at 4 °C overnight. After TBST washing, membranes were incubated with secondary antibody (Li-Cor bioscience, Lincoln, NE) (1:8000) for 1 hour at room temperature. After TBST washing, bands were analyzed using an image-processing program (Li-Cor Odyssey).

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed with EZ CHIP kit (Millipore, Billerica, MA), according to manufacturer's protocol. Purified precipitated DNA was used as the template for qPCR and primers used were listed in Supplemental Table 1.

Nuclear and cytoplasmic protein extraction

Nuclear and cytoplasmic protein extraction was performed with NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Waltham, MA) in accordance with the manufacturer's protocol. Histone 3 (CST, Danvers, MA) served as an internal control of nuclear protein. Actin (Santa Cruz, CA) served as internal control for cytoplasmic protein.

siRNA-mediated gene knockdown

siTFEB (AGACGAAGGUUCAACAUCA), siTFEB #2 (CUACAUCAAUCCUGAAAUG), and siHO1(pool of GGCAGAGGGUGAUAGAAGA, ACACUCAGCUUUCUGGUGG, AGAGAAUGCUGAGUUCAUG, and GAGGAGAUUGAGCGCAACA) were from (Dharmacon, Lafayette, CO). siAtg5

(SignalSilence Atg5 siRNA I, #6345) was from CST (Danvers, MA). siSOD2 (CGCUUACUACCUUCAGUAGtt) was from Ambion (Austin, TA). Endothelial cells were transfected with siRNA or non-targeting siRNA (Ambion In Vivo Negative Control #1 siRNA, Thermo Scientific, Waltham, MA) (20nM) using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol.

Plasmid construction and transfection

Desired DAN fragments of HO1 promoter (-483-+14) and SOD2 intron2 (787-1769) from human genome were PCR-amplified and cloned into pGL4.11 luciferases reporter vector (Promega, Madison, WI). Mutation of the putative binding site was performed using Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). TFEB overexpression plasmid was generated by cloning human TFEB coding region to pcDNA3.1 mammalian expression vector (Thermo Scientific, Waltham, MA). All PCR products were verified by DNA sequencing. BAECs were co-transfected with plasmid at 70-80% confluence, using lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the suggested protocol. Promoter activity was detected by Firefly luciferase and normalized against Renilla luciferase activity.

Construction of adenoviruses

Adenoviruses encoding GFP and human TFEB were generated by cloning the coding region of human TFEB and control GFP into AdTrack-CMV (Aglient Technologies, Santa Clara, CA). Next, the coding region was cloned from Ad-track into Ad-Easy vector by homologous recombination in *Escherichia coli*. The adenovirus encoding LacZ and human TFEB were generated by cloning the coding region of TFEB and control LacZ into PCR8/GW/TOPO TA vector (Invitrogen, Carlsbad, CA). The adenovirus encoding flag-TFEB was generated by inserting flag tag to the N-terminal of human TFEB coding region. Next, the sequence was

cloned from Entry Vector to the pAd/CMV/V5-DEST Vector (Invitrogen, Carlsbad, CA) by LR recombination. The adenoviruses were packaged in HEK293 cells and purified by CsCl2 density gradient ultracentrifugation. Adenovirus titration was determined by the Adeno-XTM quantitative PCR titration kit (Clontech, CA, USA).

Statistics

Data are presented as mean ± SEM. Student t test or 1-way ANOVA followed by Holm-Sidak test was used to analyze data. A $p<0.05$ was considered as statistical significance. All results were represented from at least 3 independent experiments.

Results

TFEB is a shear stress-responsive gene

Shear stress is a biomedical force imposed on endothelium by blood flow, which is a critical factor regulating vascular homeostasis[11]. Laminar shear stress modules regional endothelial inflammation, nitric oxide production, reactive oxygen species, and permeability and determines the susceptibility to atherosclerotic plaque buildup[110, 111]

Shear stress affects a diverse spectrum of the process in endothelial cells, including inflammation, proliferation, and survival[112, 113]. In contrast to oscillatory shear stress, which promotes atherosclerosis, laminar shear stress is protective against atherosclerosis[114]. In human umbilical vein endothelial cells (HUVECs), TFEB abundance was significantly increased by 48-hour laminar shear stress compared with static conditions or oscillatory shear stress both at mRNA (5.19±0.76 fold compared to static conditions and 5.11±0.76 fold compared to oscillatory shear stress) and protein (2.38±0.17 fold compared to static conditions and 1.78±0.20 fold compared to oscillatory shear stress) (Fig. 2.1 A-B). Indeed, we found that laminar shear stress-induced TFEB nuclear translocation (Fig. 2.1 C-D), and decreased mammalian target of

rapamycin (mTOR) activity with impaired phosphorylation at $Thr^{37/46}$ of 4EBP1 (substrate of mTOR kinase) (Fig. 2.1 E). To determine the TFEB abundance pattern *in vivo*, we performed en face staining of TFEB on different sites of the rabbit aorta [115, 116]. Our results showed that atherosclerosis-resistant regions (greater curvature and descending aorta) showed higher TFEB protein abundance, compared to atherosclerosis-prone areas (less curvature) (Fig. 2.1 F). In addition, we determined whether TNFα treatment affects TFEB abundance. Together, our data suggest that *TFEB* is a shear stress-responsive gene that could have a critical role in endothelial cell biology.

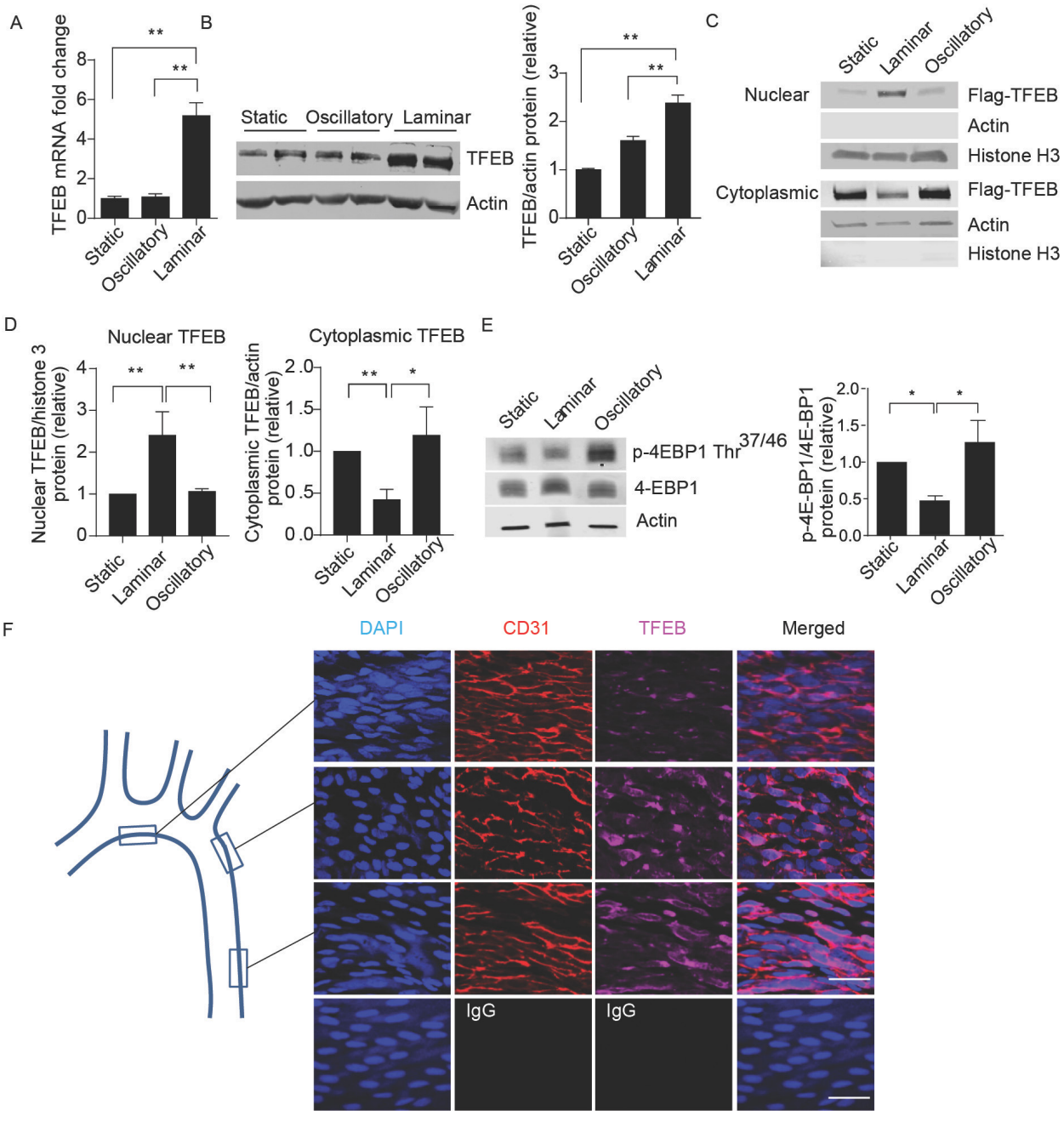

A
TFEB mRNA fold change
Static
Oscillatory
Laminar
B
Static Oscillatory Laminar
TFEB
Actin
TFEB/actin protein (relative)
C
Static
Laminar
Oscillatory
Nuclear
Flag-TFEB
Actin
Histone H3
Cytoplasmic
Flag-TFEB
Actin
Histone H3
D
Nuclear TFEB
Nuclear TFEB/histone 3 protein (relative)
Cytoplasmic TFEB
Cytoplasmic TFEB/actin protein (relative)
E
p-4EBP1 Thr37/46
4-EBP1
Actin
p-4E-BP1/4E-BP1 protein (relative)
F
DAPI
CD31
TFEB
Merged
IgG
IgG

Figure 2.1 Laminar shear stress increased TFEB mRNA and protein abundance.

(A and B) HUVECs were exposed to static conditions, oscillatory shear stress, or laminar shear stress for 48 hours. (A) TFEB mRNA was determined by quantitative polymerase chain reaction (qPCR) and normalized against 18S RNA. (B) TFEB protein was determined by Western blot. (C and D) HUVECs were infected with adenovirus encoding Flag-TFEB. After 4-hour shear stress treatment, TFEB protein abundance was determined in the cytoplasmic and nuclear fractions. (E) After 4-hour shear stress treatment, phosphorylated (p) and total 4E-BP1 protein in HUVECs was determined by (F) TFEB protein abundance in different areas of the rabbit aorta were determined by en face immunostaining. Data are representative of three independent experiments.

Overexpression of TFEB inhibits endothelial cell inflammation

Laminar shear stress exhibits anti-inflammatory effects in endothelial cells both *in vitro* [117] and *in vivo*[114]. Endothelial cell inflammation is characterized by the induction of various adhesion molecules and cytokines [91]. TFEB overexpression suppressed the expression of E-selectin (*SELE*), monocyte chemoattractant protein 1 (*MCP1*) and vascular cell adhesion molecule 1 (*VCAM1*) mRNA in HUVECs in response to the proinflammatory stimuli TNFα, interleukin 1β (IL1β) or lipopolysaccharide (LPS) (Fig. 2.2 A). TFEB also significantly decreased E-selectin and VCAM1 protein abundance by 49±12% and 46±11%, respectively (Fig. 2.2 B). The anti-inflammatory function of TFEB was not limited to HUVECs, as TFEB also exerted a similar anti-inflammatory effect in primary human coronary artery endothelial cells (HCAECs) (Fig. 2.2 C-D). Thus, TFEB exerted a potent inhibitory effect on inflammation in the presence of a variety of proinflammatory factors.

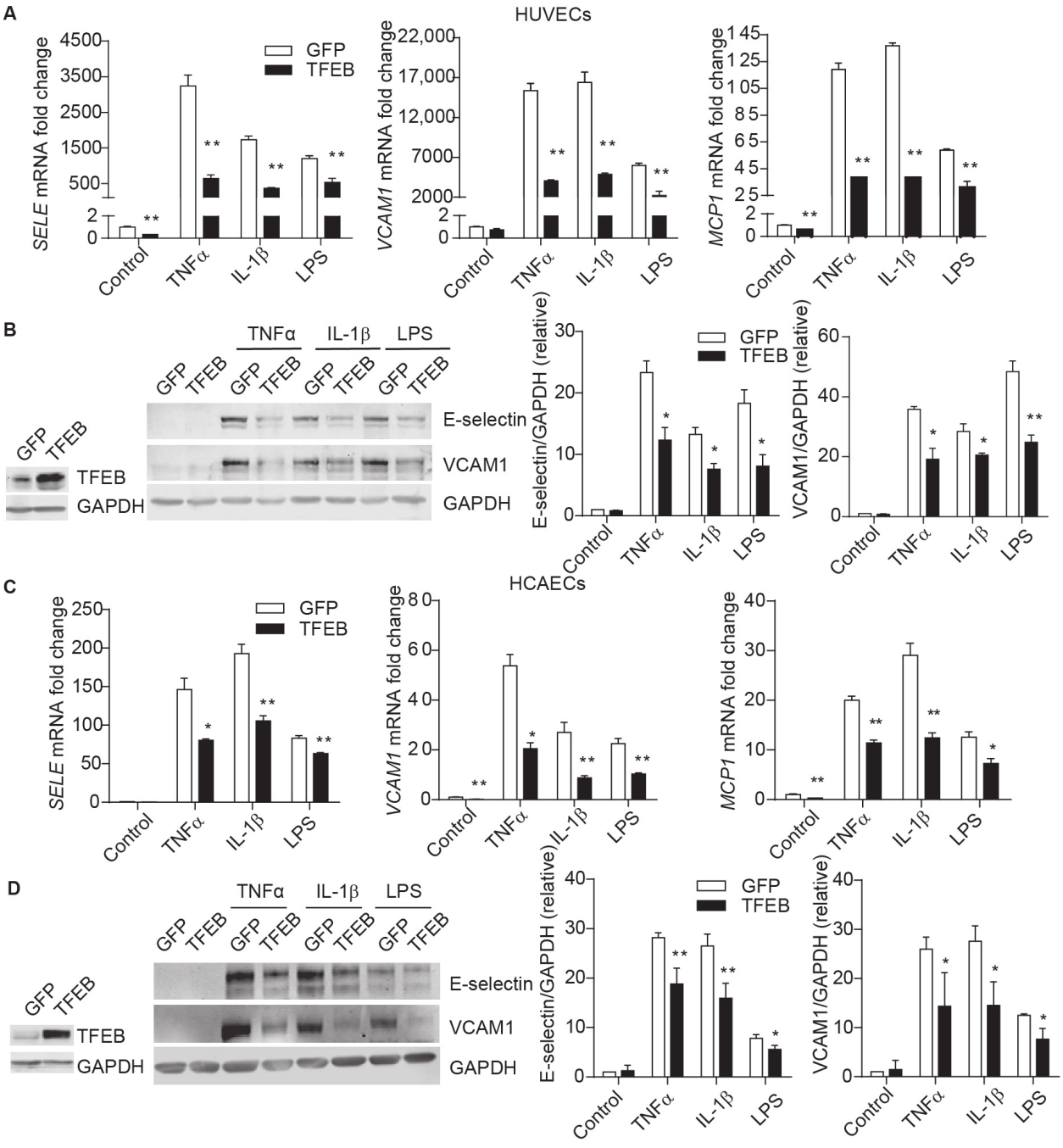

A
HUVECs
SELE mRNA fold change
VCAM1 mRNA fold change
MCP1 mRNA fold change
GFP
TFEB
Control
TNFα
IL-1β
LPS
B
TNFα
IL-1β
LPS
GFP
TFEB
E-selectin
VCAM1
GAPDH
E-selectin/GAPDH (relative)
VCAM1/GAPDH (relative)
C
HCAECs
D

Figure 2.2 TFEB potently inhibits inflammation in endothelial cells.

(A-B) HUVECs were infected with adenovirus encoding GFP or human TFEB and treated with tumor necrosis factor α (TNFα), interleukin 1β (IL1β) or lipopolysaccharide (LPS). (A) *SELE, VCAM1* and *MCP1* mRNA were determined by qPCR. (B) TFEB, E-selectin and VCAM1 protein were determined by Western blot. Band densities were quantitatively analyzed and normalized against GAPDH. (C-D) HCAECs were infected with Ad-GFP or Ad-TFEB and treated with TNFα, IL1β or LPS. (C) *SELE, VCAM1* and *MCP1* mRNA were determined by qPCR. (D) TFEB, E-selectin and VCAM1 protein were determined by Western blot. Data were from 3 independent experiments and presented as mean ± SEM. *$p<0.05$ **$p<0.01$.

TFEB knockdown aggravates inflammation in endothelial cells

To further elucidate the essential role of TFEB in regulating inflammation, we used a small interfering RNA (siRNA) strategy and achieved a >90% knockdown of TFEB mRNA and protein in HUEVCs (Fig. 3 A). The knockdown of TFEB increased the expression of *SELE*, *VCAM1*, *MCP1,* and *IL6* by 2.67±0.18, 8.44±2.2, 1.42±0.08 and 2.26±0.35 fold, respectively (Fig. 2.3 B) and increased the protein abundance of E-selectin and VCAM1 by 0.65±0.12, 0.3±0.07 fold, respectively, upon TNFα stimulation (Fig. 2.3 C). To determine whether TFEB mediates the anti-inflammatory effect of laminar shear stress in endothelial cells, we conducted TFEB knockdown prior to shear stress treatment (Fig. 2.3 D-E). We found that laminar flow could suppress pro-inflammatory adhesion molecules in the presence of TNFα in the control cells. However, TFEB knockdown significantly attenuated the anti-inflammatory effect of laminar flow on endothelial cells (Fig. 2.3 D-E). Taken together, our results suggest that endogenous TFEB suppresses inflammation in response to pro-inflammatory stimuli and mediates the protective effect of laminar shear stress in endothelial cells.

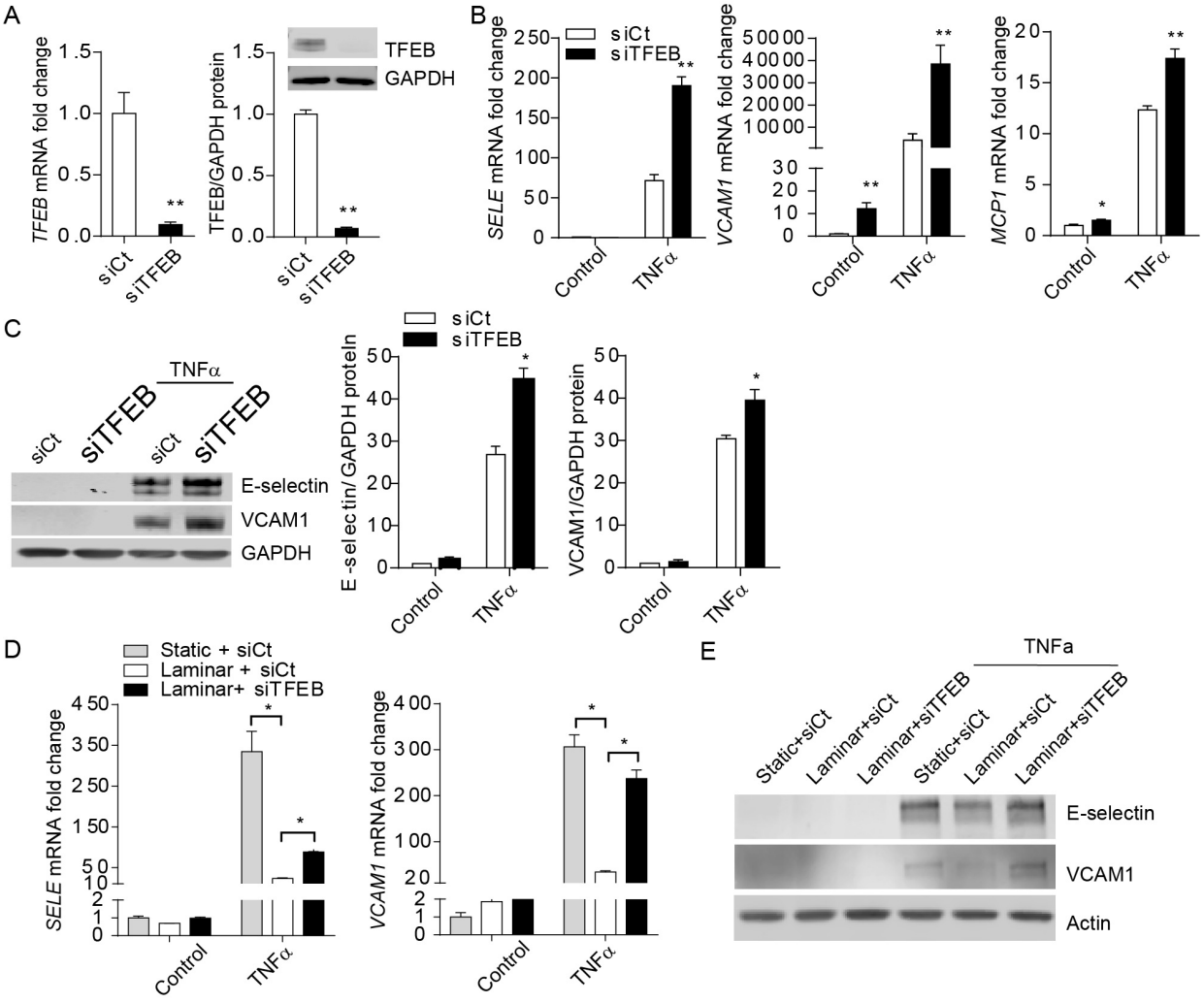


Figure 2.3 TFEB knockdown aggravates inflammation in endothelial cells.

HUVECs were transfected with siCt or siTFEB before treatment with TNFα. (A) TFEB knockdown efficiency was determined by qPCR and Western blot. (B) *E-selectin, VCAM1, MCP1* and *IL6* mRNA were determined by qPCR. (C) E-selectin and VCAM1 protein were determined by Western blot. (D-E) HUVECs were transfected with small interfering RNA (siRNA)-control (siCt) or siRNA-TFEB (siTFEB) before exposure to static condition or laminar shear stress for 24 hours, and then treated with TNFα. VCAM-1 and E-selectin abundance was determined by qPCR (D) and Western blot (E). Data were from 3 independent experiments and presented as mean ± SEM. *p<0.05; **p<0.01.

TFEB reduces intracellular ROS in HUVECs

Oxidative stress induced by ROS in endothelial cells triggers the production of proinflammatory molecules and cytokines [94, 96, 108, 118, 119]. We found that overexpression of TFEB reduced ROS production, while TFEB knockdown augmented ROS production, both basally and in response to TNFα treatment using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) assay (Fig. 2.4 A-B). We also repeated the results using luminol chemiluminescence as an additional method to determine ROS concentrations (Fig. 2.4 C-E). We also measured superoxide by dihydroethidium (DHE) fluorescence assay in TFEB overexpressing and knockdown endothelial cells. Our data show that TFEB significantly inhibited intracellular superoxide in endothelial cells (Fig. 2.4 F-G).

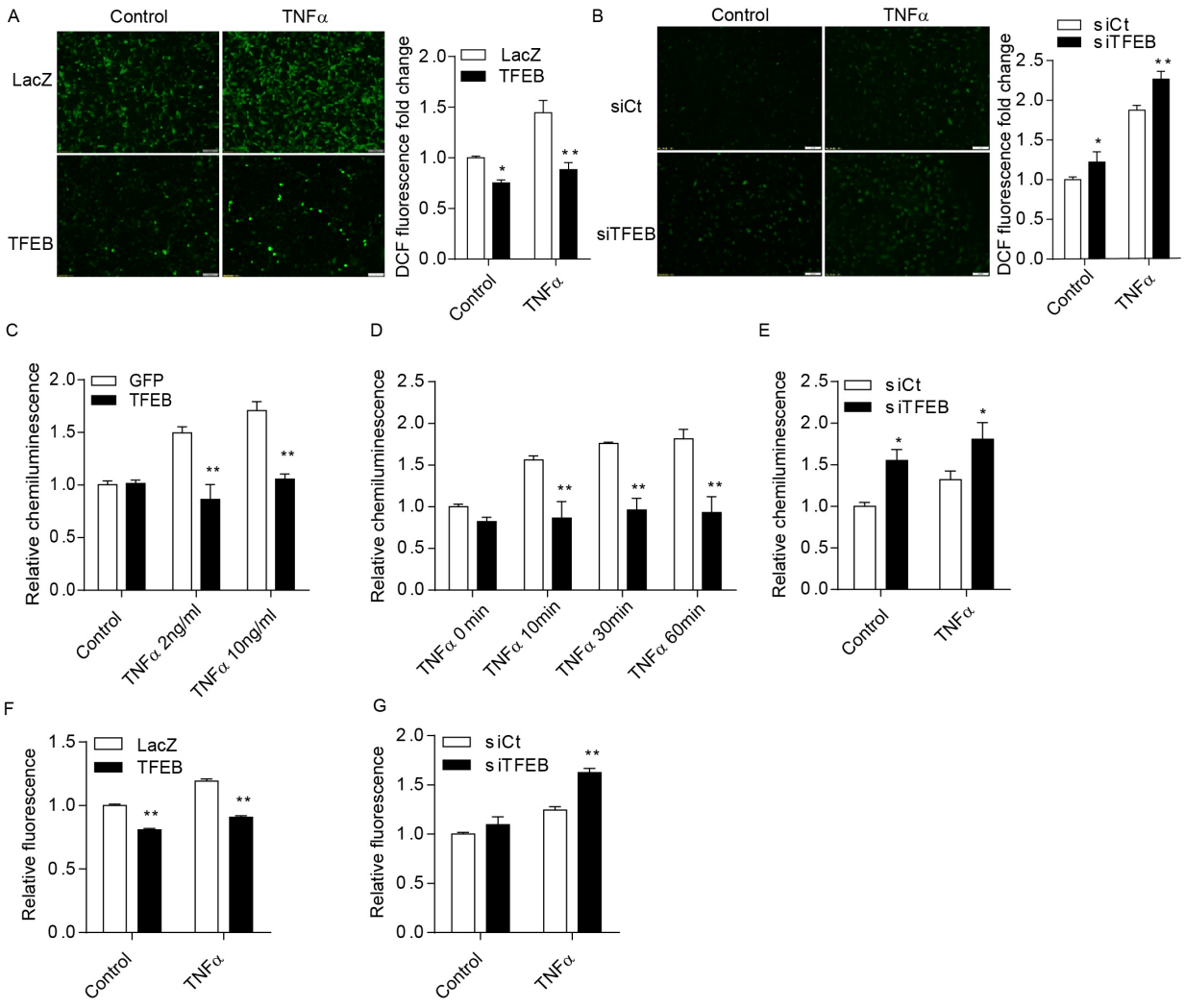


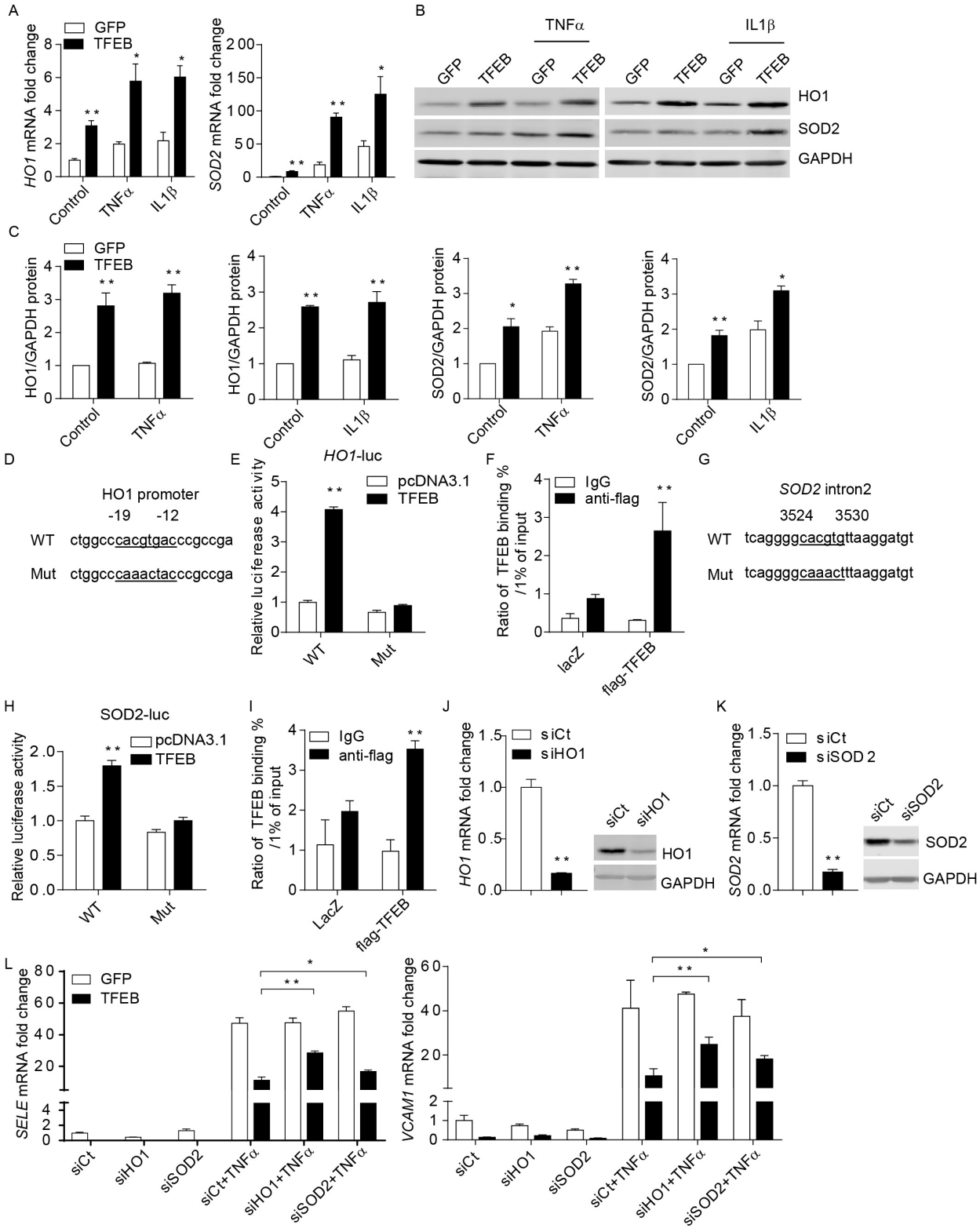
Figure 2.4 TFEB reduces intracellular ROS concentrations in HUVECs.

(A-B) HUVECs were infected with (A) Ad-LacZ or Ad-TFEB or (B) transfected with siCt or siTFEB. Endothelial cells were loaded with 2', 7'-dichlorodihydrofluorescin diacetate (DCFH-DA) to probe for reactive oxygen species (ROS) before treatment with TNFα. Fluorescence was determined by fluorescence microscopy (left panel, representative image of 3 independent experiments) or microplate reader (right panel). (C-D) HUVECs were infected with Ad-GFP or Ad-TFEB before treatment with TNFα for the indicated dosages (C) and time (D), followed by luminol loading. Luminescence was detected with a luminometer. (E) HUVECs were transfected with siCt or siTFEB, followed by TNFα treatment and luminol loading. Luminescence was detected with a luminometer. (F) HUVECs were infected with Ad-GFP or Ad-TFEB or (G) transfected with siCt or siTFEB before treatment with TNFα and DHE loading. Fluorescence was determined by microplate reader. Data were from 3 independent experiments and presented as mean ± SEM. *$p<0.05$; **$p<0.01$. Scale bar: 100μm.

TFEB increases the transcription of antioxidant genes

In endothelial cells, enzymatic and non-enzymatic antioxidant systems prevent cells from oxidative damage. Major enzymatic antioxidants include superoxide dismutase (SOD), Catalase, glutathione peroxidase (GPX1), heme oxygenase 1 (HO1), thioredoxin (TXN1) and peroxiredoxin (PRX) [95, 120]. HO1 and SOD2 protect against endothelial dysfunction and atherosclerosis. TFEB overexpression increased both mRNA and protein for HO1 and SOD2 (Fig. 2.5 A-C). TFEB binds to a palindromic 10–base pair (bp) GTCACGTGAC motif and induces the transcription of its target genes [121]. We found a putative TFEB binding site located at -19/-12 in the *HO1* promoter (Fig. 2.5 D). A transcriptional activity reporter containing this binding site from the *HO1* promoter displayed higher luciferase activity in TFEB overexpressing cells when compared to cells expressing endogenous TFEB (vector control), an effect that was abolished by mutation of this motif (Fig. 2.5 E). Chromatin Immunoprecipitation (ChIP) assays also demonstrated that TFEB bound the *HO1* promoter in the area that harbors this motif (Fig. 2.5 F). We also found a putative TFEB binding site in the intron2 region of *SOD2* (Fig. 2.5 G), which is consistent with evidence that intron2 harbors several elements that regulate *SOD2* expression [122-124]. Luciferase assays confirmed that the reporter driven by this region harboring the binding site was activated by TFEB overexpression, an effect that was lost by mutation of the binding site (Fig. 2.5 H) and TFEB bound to this site in *SOD2* intron 2, as determined by ChIP assay (Fig. 2.5 I). To determine whether SOD2 or HO1 are necessary for TFEB-dependent inhibition of inflammation in endothelial cells, we knocked down HO1 and SOD2 in the TFEB overexpressing endothelial cells. We found that HO1 knockdown largely attenuated the anti-inflammatory effect of TFEB, while SOD2 knockdown had a modest effect on the TFEB regulation of the inflammatory status (Fig. 2.5 J-L). Together, these results indicate that TFEB

directly increases the transcription of the *HO1* and *SOD2* genes. Although other anti-inflammatory signaling pathways cannot be excluded, enhanced anti-oxidative capacity through increasing HO1 and SOD2 could contribute in part to the anti-inflammatory effect of TFEB in endothelial cells.


A
GFP
TFEB
HO1 mRNA fold change
SOD2 mRNA fold change
Control
TNFα
IL1β
B
TNFα
IL1β
GFP
TFEB
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SOD2
GAPDH
C
HO1/GAPDH protein
SOD2/GAPDH protein
D
HO1 promoter
-19 -12
WT ctggcccacgtgaccgccga
Mut ctggcccaaactaccgccga
E
HO1-luc
pcDNA3.1
TFEB
Relative luciferase activity
F
IgG
anti-flag
Ratio of TFEB binding % /1% of input
lacZ
flag-TFEB
G
SOD2 intron2
3524 3530
WT tcaggggcacgtgttaaggatgt
Mut tcaggggcaaacttaaggatgt
H
SOD2-luc
I
J
siCt
siHO1
K
siSOD2
L
SELE mRNA fold change
VCAM1 mRNA fold change
siHO1+TNFα
siSOD2+TNFα

Figure 2.5 TFEB increases mRNA and protein abundance of anti-oxidant genes.

(A-C) HUVECs were infected with Ad-GFP or Ad-TFEB before treatment with TNFα or IL1β. (A) Heme oxygenase 1 *(HO1)* and superoxide dismutase 2 *(SOD2)* mRNA was determined by qPCR. (B) HO1 and SOD2 protein were determined by Western blot. (C) Band densities in (B) were quantitatively analyzed and normalized against GAPDH. (D) Wild-type and mutant *HO1* promoter or (G) *SOD2* intron2 region was cloned into the pGL4.11 luciferase reporter vector. (E-H) Luciferase activity was determined in AD-293 cells transfected with *HO1* pGL4.11 (E) or *SOD2* pGL4.11 plasmids (H), together with pcDNA 3.1 empty vector or pcDNA3.1 encoding human TFEB. (F&I) HUVECs were infected with Ad-LacZ or Ad-flag-TFEB. The binding of TFEB to *HO1* promoter (F) or *SOD2* intron2 region (I) was determined by ChIP assay (J-L) HUVECs were transfected with siCt, siHO1 or siSOD2 before TNFα treatment. (J&K) HO1 and SOD2 knockdown efficiency were determined by qPCR and Western blot. (L) *SELE* and *VCAM1* mRNA was determined by qPCR. Date were from 3 independent experiments and presented as mean ± SEM. *$p<0.05$; **$p<0.01$

TFEB overexpression did not inhibit the NF-κB pathway in ECs.

The Nf-κb pathway is one of the most important inflammatory pathways[125]. However, our data suggested that TFEB overexpression did not inhibit the TNFα-induced activation of the NF-κB pathway in endothelial cells, as assessed by both NF-κB element driving luciferase (Fig. 2.6 A), IκBα degradation (Fig. 2.6 B) and p65 nuclear translocation (Fig. 2.6 C).

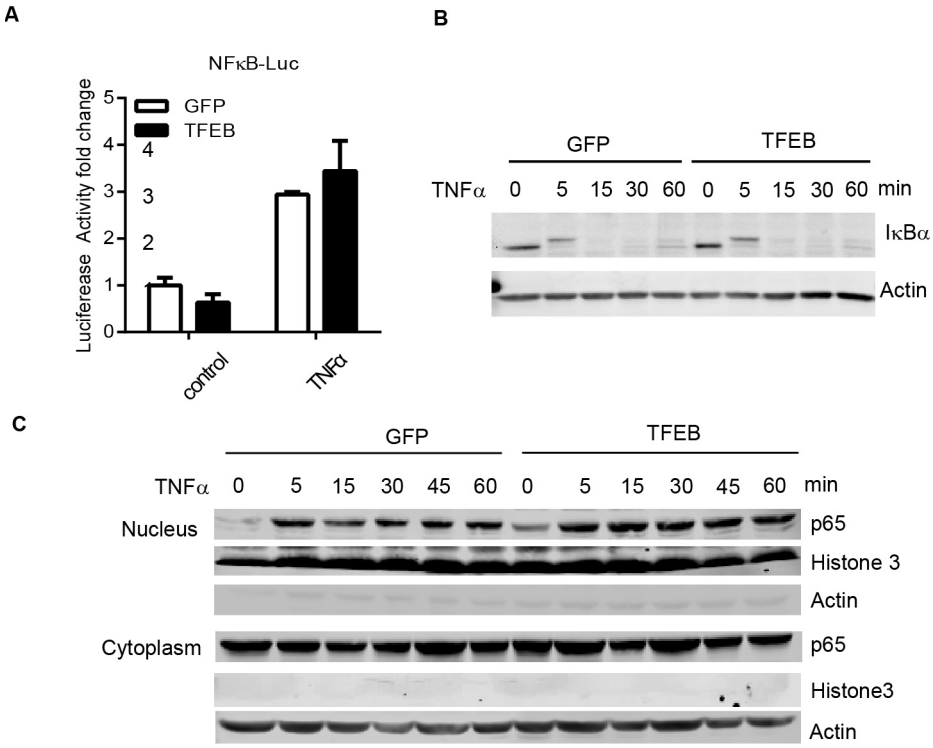

Figure 2.6 TFEB did not inhibit NF-kB pathway in endothelial cells.

(A) Bovine aortic endothelial cells (BAECs) were transfected with Nuclear Factor κB (NF-κB)-luciferase for 24 hours and then transfected with Ad-GFP or Ad-TFEB. Promoter activity was determined after TNFα treatment for 12 hours by Firefly luciferase and normalized against Renilla luciferase activity. Data were from 3 independent experiments and presented as mean ± SEM. (B-C) HUVECs were infected with Ad-GFP or Ad-TFEB and then treated with TNFα for the indicated time. Data is representative of 3 independent experiments. (B) IκBα protein was determined by Western blot. (C) After isolation of nuclear and cytoplasmic protein extracts, p65 protein was determined by Western blot in the different fractions.

Inhibition of autophagy did not diminish the inhibitory effect of TFEB on endothelial cell inflammation

Autophagy is an evolutionarily conserved process that degrades protein and damaged organelles. TFEB is a master regulator of lysosomal biogenesis and autophagy in various cell types [100, 126]. We determined the expression of autophagy genes in TFEB overexpressing HUVECs. TFEB significantly increased the mRNA abundance of autophagy related 3 (*ATG3*), autophagy related 9B (*ATG9B*), sequestosome 1 (*SQSTM1*), lysosomal associated membrane protein 1 (*LAMP1*), ATPase H+ transporting V1 Subunit H (*ATP6V1H*), glucosamine (N-Acetyl)-6-sulfatase (*GNS*), galactosidase alpha (*GLA*) and hexosaminidase A (*HEXA*) (Fig. 2.7 A). TFEB overexpression also increased ATG3, microtubule-associated protein 1A/1B-light chain 3-I (LC3-I) and LC3-II protein abundance (Fig. 2.7 B).

We examined whether the anti-inflammatory effect of TFEB in endothelial cells was autophagy dependent. Autophagy related 5 (ATG5) is required for the formation of autophagosomes [127]. In HUVECs with ATG5 knockdown (Fig. 2.7 C), TFEB overexpression still potently decreased the mRNA of *SELE*, *VCAM1,* and *MCP1* to a comparable extent as in controlled endothelial cells (Fig. 2.7 D). In addition, pharmacological inhibition of autophagy by pretreatment with 3-Methyladenine (3-MA) [128], chloroquine (CQ) [129] or bafilomycin A1 [130] for either 30 minutes or 16 hours, did not attenuate the inhibitory effect of TFEB on inflammation in endothelial cells (Fig. 2.7 E-F). Therefore, TFEB inhibition of endothelial cell inflammation may be independent of its activation of the canonical autophagy pathway.

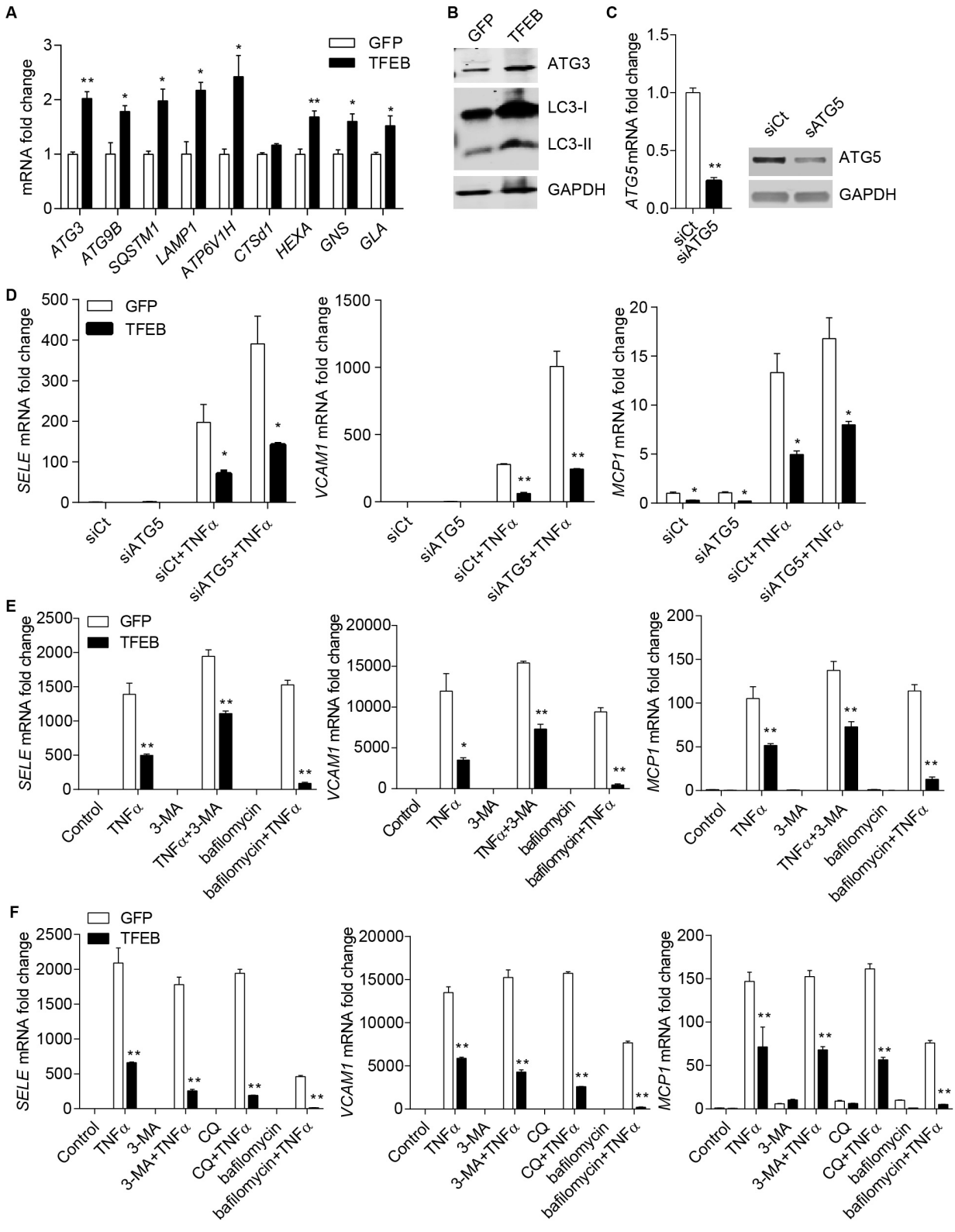
A
mRNA fold change
GFP
TFEB
ATG3
ATG9B
SQSTM1
LAMP1
ATP6V1H
CTSd1
HEXA
GNS
GLA
B
GFP
TFEB
ATG3
LC3-I
LC3-II
GAPDH
C
ATG5 mRNA fold change
siCt
siATG5
siCt
sATG5
ATG5
GAPDH
D
SELE mRNA fold change
VCAM1 mRNA fold change
MCP1 mRNA fold change
GFP
TFEB
siCt
siATG5
siCt+TNFα
siATG5+TNFα
E
SELE mRNA fold change
VCAM1 mRNA fold change
MCP1 mRNA fold change
GFP
TFEB
Control
TNFα
3-MA
TNFα+3-MA
bafilomycin
bafilomycin+TNFα
F
SELE mRNA fold change
VCAM1 mRNA fold change
MCP1 mRNA fold change
GFP
TFEB
Control
TNFα
3-MA
3-MA+TNFα
CQ
CQ+TNFα
bafilomycin
bafilomycin+TNFα

Figure 2.7 Inhibition of autophagy cannot attenuate the inhibitory effect of TFEB on inflammation in endothelial cells.

(A-B) HUVECs were infected with Ad-GFP or Ad-TFEB. (A) mRNA for autophagy and lysosome biogenesis related genes was determined by qPCR. (B) ATG3, LC3-I and LC3-II protein were determined by Western blot (n=2 blots). (C-D) HUVECs were transfected with siCt or si-autophagy related 5 (siATG5) and then infected with Ad-GFP or Ad-TFEB, followed by treatment with TNFα. (C) ATG5 knockdown efficiency was determined by qPCR and Western blot. (D) *SELE, MCP1,* and *VCAM1* mRNA were determined by qPCR. (E) HUVECs were infected with Ad-GFP or Ad-TFEB and pretreated with the autophagy inhibitors 3-MA or bafilomycin A1 for 30 min before treatment with TNFα. (F) HUVECs were infected with Ad-GFP or Ad-TFEB and pretreated with the autophagy inhibitors 3-MA, CQ or bafilomycin A1 for 16 hours before treatment with TNFα. *SELE, VCAM1,* and *MCP1* mRNA were determined by qPCR. Data were from 3 independent experiments and presented as mean ± SEM. *$p<0.05$ **$p<0.01$.

Generation of EC-TFEB transgenic mouse.

We generated a transgenic mouse that overexpressed TFEB under the mTie2 promoter in endothelial cells (EC-TFEB) (Fig. 2.8 A-B). The overexpression of TFEB is confirmed by immunofluorescence (CD31 as an EC marker) (Fig. 2.8 C). We also assessed the TFEB protein in the peritoneal macrophage and bone marrow hematopoietic cells and did not find a significant change (Fig. 2.8D-E).

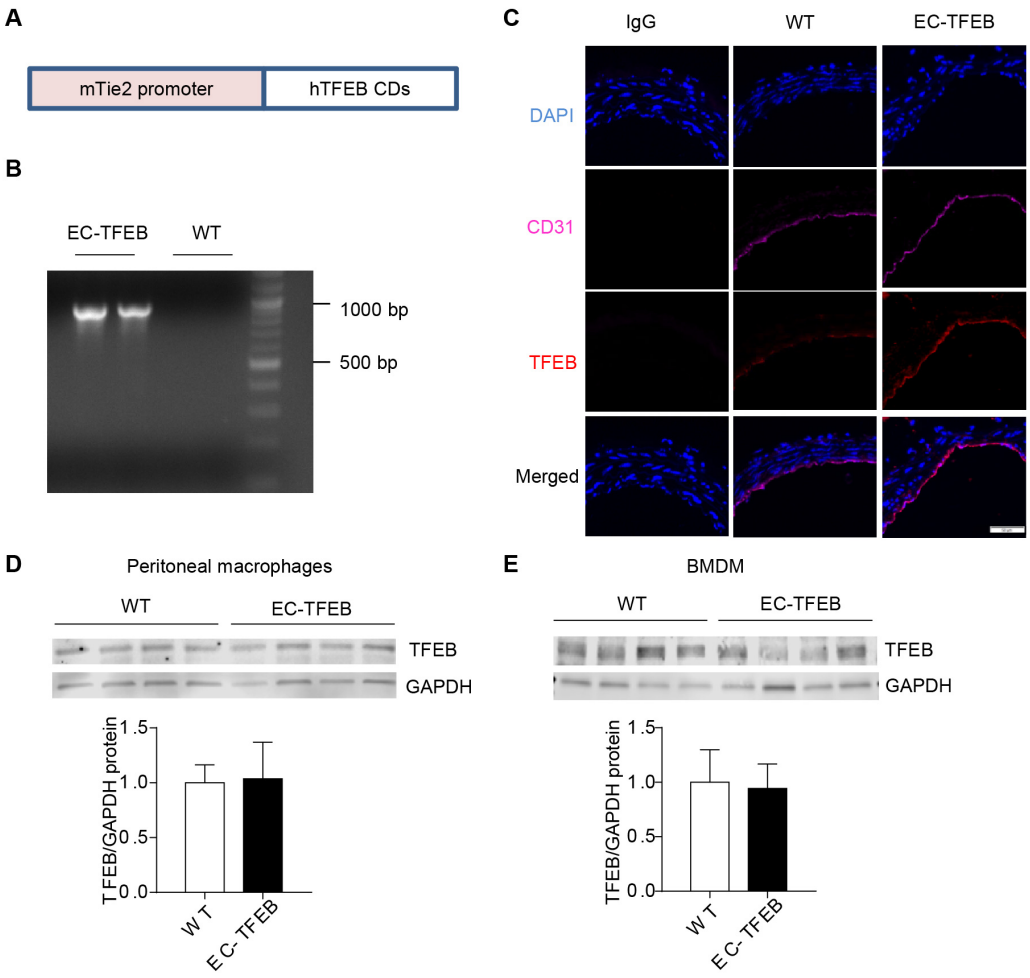

Figure 2.8 Characterization of EC-TFEB transgenic mice.

(A) Schematic of *EC-TFEB* transgene (coding region sequence, CDs). (B) Genotyping results of *EC-TFEB* transgenic (Tg) and control *wild-type* mice (in 2% agarose gel). (C) Immunofluorescence for cluster of differentiation 31 (CD31) (Ex/Em = 650/665) and TFEB (Ex/Em = 590/617) in the thoracic aorta from *EC-TFEB* and littermate wild-type mice. Normal rabbit IgG was used as a negative control. Data are representative of 3 independent experiments. Scale bar: 50 μm. (D-E) Peritoneal macrophages and bone marrow hematopoietic cells were isolated from *EC-TFEB* and *wild-type* mice. TFEB protein abundance in these cells was determined by Western blot, n=4 for each group.

EC-TFEB transgene inhibits endothelial inflammation and reduces atherosclerosis development in mice

Endothelial cell activation is an early event step of atherogenesis and inhibition of this step can largely attenuate the development of atherosclerosis [131]. The adhesion of leukocytes to vascular endothelium is a hallmark of endothelial cell inflammation. After administration of LPS, *EC-TFEB* mice demonstrated significantly decreased leukocyte rolling and adhesion on endothelial cells by 68±19% and 59±20%, respectively, in the vessels in the cremaster muscles. Comparable to the *in vitro* study, administration of CQ did not abolish this phenotype in *EC-TFEB* mice when compared with *wild-type* mice (Fig. 2.9 A-B). Furthermore, compared with littermate control mice, the aortas from *EC-TFEB* mice showed decreased VCAM1 abundance assessed by immunostaining after LPS administration (Fig. 2.9 C). Consistent with these findings, *SELE* and *VCAM1* mRNA abundance were significantly decreased in the aortas of *EC-TFEB* mice after LPS administration when compared with control mice (Fig. 2.9 D). To determine if TFEB overexpression in endothelial cells prevented atherosclerosis development, we crossbred *EC-TFEB* mice with atherosclerosis-prone *ApoE*$^{-/-}$ mice and mice were fed a high-cholesterol diet. Measurement of the atherosclerotic lesion areas revealed that *EC-TFEB*/*ApoE*$^{-/-}$ mice exhibited significantly decreased atherosclerotic lesion formation by 46±16% ($p<0.01$), compared with littermate *ApoE*$^{-/-}$ mice (Fig. 2.9 E-F). These results indicate that TFEB is a critical suppressor of endothelial cell inflammation and atherosclerosis *in vivo*.

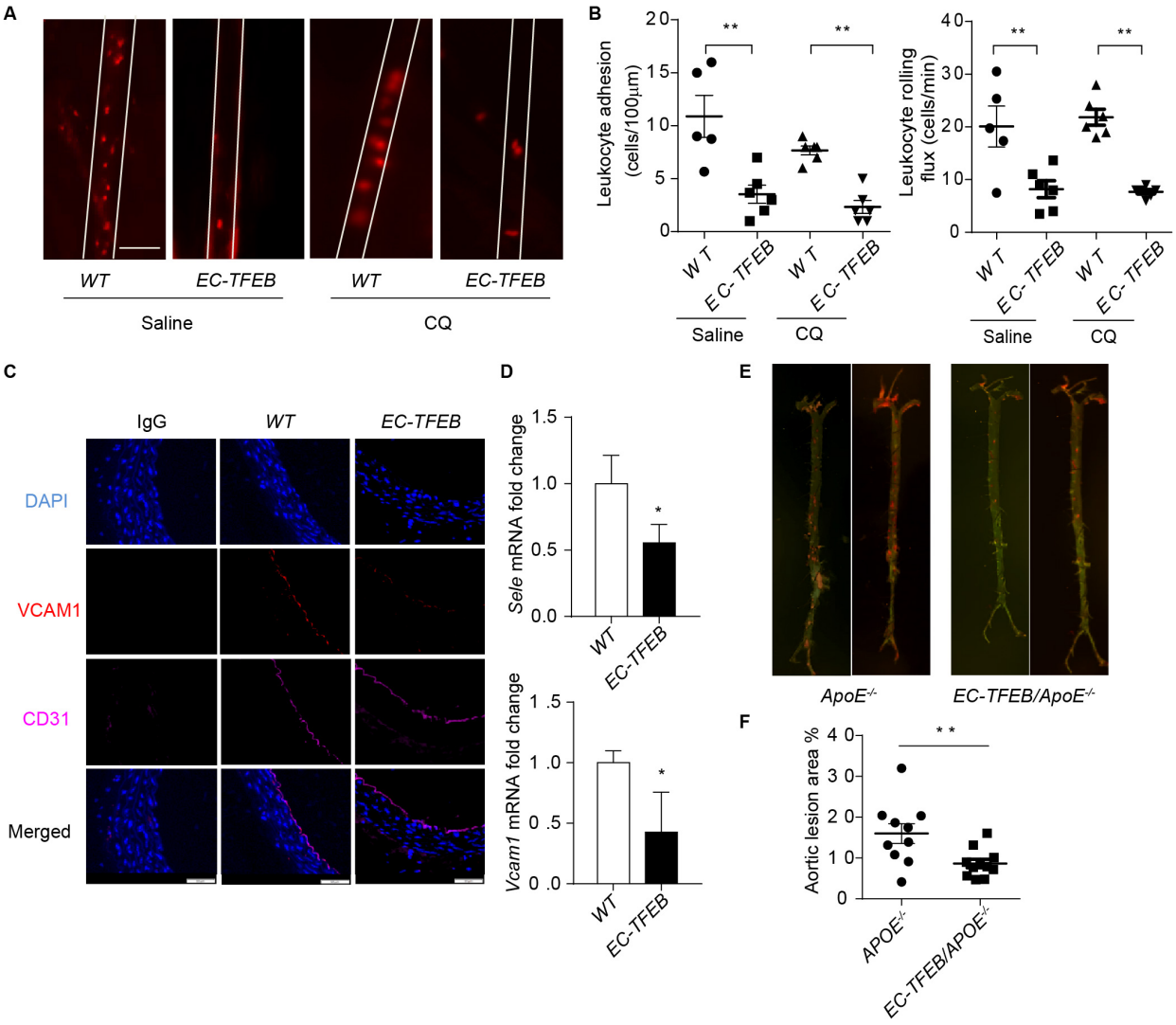

Figure 2.9 Fig. 2.9 EC-TFEB transgene inhibits endothelial cell inflammation and reduces atherosclerosis development.

(A-B) *EC-TFEB* transgenic and littermate *WT* mice were treated with saline or CQ for 7 days followed by administration of LPS. (A) Leukocyte recruitment was analyzed with intravital microscopy. Scale bar: 50μm. (B) The adhesion and rolling of leukocytes on vascular walls were quantitatively analyzed. N=5-6 mice for each group. (C) Immunostaining for VCAM1 (Ex/Em = 590/617) and CD31 (Ex/Em = 650/665) in the thoracic aortas from *EC-TFEB* and littermate *WT* mice after LPS administration. Normal rabbit IgG was used as a negative control. (D) *Sele* and *Vcam1* mRNA in the aortas from *EC-TFEB* and littermate control mice after LPS injection was determined by qPCR (normalized against 18S RNA). (E-F) *EC-TFEB/ ApoE*$^{-/-}$ and *ApoE*$^{-/-}$ mice were fed a high cholesterol diet. (E) En face analysis of atherosclerotic lesions in the aortic tree was performed after oil-red O staining. (F) The area of atherosclerotic lesions was quantified. N=10-11 mice for each group. Data were presented as mean ± SEM. *p<0.05; **p<0.01. Scale bar: 50μm.

The EC-TFEB transgene does not affect plasma lipid profile in ApoE$^{-/-}$ mice

Plasma samples from the two groups showed no significant differences in total cholesterol, high-density lipoprotein cholesterol (HDL-c), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-c) concentrations (Fig. 2.10).

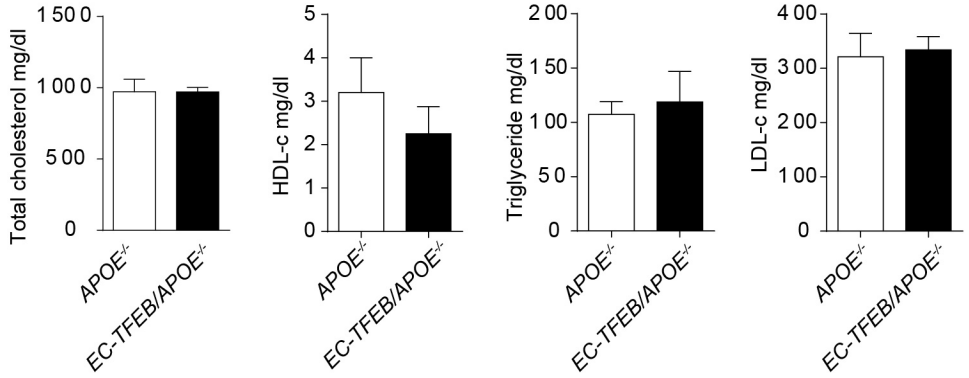

Figure 2.10 The *EC-TFEB* transgene did not alter plasma lipid profile in *ApoE*$^{-/-}$ mice.

EC-TFEB/ApoE$^{-/-}$ and *ApoE*$^{-/-}$ mice were placed on a high cholesterol diet for 8 weeks. Plasma total cholesterol, HDL-c, TG and LDL-c were measured. N=5-6 mice for each group. Data are presented as mean ± SEM.

Discussion

CVDs are the leading cause of death in the United States[132]. Current evidence supports the central role of endothelial cell inflammation in atherosclerosis[133]. Physiological laminar shear stress is well-recognized as protective by inhibition of endothelial cell inflammation both *in vitro* and *in vivo*[134]. The finding that endothelial TFEB is significantly induced by laminar shear stress, compared with the static condition or oscillatory shear stress prompted us to investigate the relationship between TFEB and endothelial cell inflammation. Our data suggest that TFEB is indispensable to modulate the inflammatory status and enhance the anti-oxidative capacity in endothelial cells (Fig. 2.11).

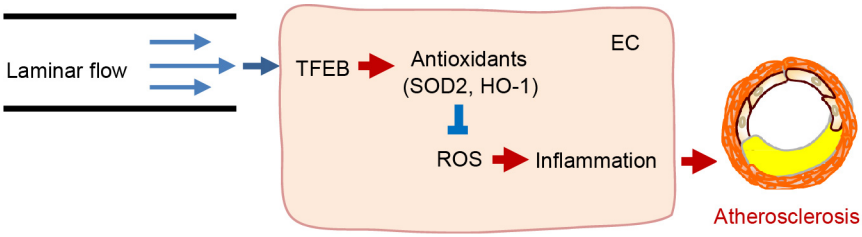

Figure 2.11 The role of TFEB in endothelial inflammation and atherosclerosis.

Diagram summarizing the findings indicating that endothelial TFEB has a protective effect on atherosclerosis through inhibition of oxidative stress and inflammation.

We found that laminar shear stress induced the TFEB nuclear translocation which is known to induce *TFEB* transcription in an auto-regulatory loop [135]. Furthermore, when TFEB is phosphorylated by mammalian target of rapamycin complex1 (mTORC1), it is retained in the cytoplasm [28, 136, 137]. We found that laminar shear stress decreased mTORC1 activity (Fig. 1E), suggesting that laminar shear stress may induce TFEB nuclear translocation in endothelial cells. Thus, enhanced TFEB nuclear translocation may account for the increased TFEB abundance induced by laminar flow in endothelial cells.

Recruitment of leukocytes to endothelial cells is an initial stage of atherogenesis and is mediated by adhesion molecules (E-selectin, VCAM1) and proinflammatory cytokines (MCP1, IL6). We used both gain-of-function and loss-of-function strategies to demonstrate that TFEB behaves as an anti-inflammatory transcription factor in endothelial cells, induced by a variety of proinflammatory stimuli. Consistent with our *in vitro* data, endothelial cell-specific TFEB transgene also inhibits endothelial cell-leukocyte adhesion *in vivo*, reinforcing that TFEB promotes an anti-inflammatory phenotype in endothelial cells. We also observed decreased atherosclerotic lesion formation in *EC-TFEB*/*ApoE*$^{-/-}$ mice, compared with littermate *ApoE*$^{-/-}$ mice, indicating that TFEB could be a potential target to prevent and treat atherosclerosis and associated CVDs.

TFEB reduces ROS in endothelial cells both at the basal level and under the condition of TNFα treatment. This phenomenon could be attributed to increased transcription of antioxidant genes in endothelial cells, including *HO1*, *SOD2,* and *TXN1*. We demonstrate that *HO1* and *SOD2* are the direct targets of TFEB in endothelial cells. ROS are key signaling molecules in the progression of inflammation [138]. Thus, enhanced TFEB-mediated anti-oxidative capacity could partially explain its anti-inflammatory function. Although the NF-κB pathway plays a critical

role in the inflammatory response in many different cell types [139], recent studies have identified that many proteins could regulate endothelial cell inflammation in an NF-κB-independent manner [108, 140, 141]. IκBα phosphorylation and degradation is induced by proinflammatory cytokines or stress stimulation, resulting in nuclear translocation of p65 to activate downstream genes. Our data revealed that neither IκBα degradation nor p65 translocation was altered in the TFEB overexpressing endothelial cells, suggesting that the role of TFEB in endothelial cells is NF-κB-independent. Based on our present study, we cannot rule out that TFEB affects other inflammatory pathways in endothelial cells, such as p38 mitogen-activated protein kinases (p38 MAPK), Janus N-terminal Kinase (JNK), Extracellular signal-regulated kinases (ERK), Janus kinase/signal transducers and activators of transcription (JAK-STAT), which will require further investigation in follow up studies.

Increased ROS have been observed virtually in every aspect of atherosclerotic plaque formation[142]. As a result, there are numerous methods to measure ROS, although each method has its own pitfalls. DCFH-DA is the most common used probe for intracellular ROS. It is cleaved and trapped in the cell. Upon oxidized, it becomes highly fluorescent product dichlorofluorescein (DCF). One major concern is that photoreduction of DCF will generate superoxide radical, amplifying the oxidative stress[143]. Luminol is a cell-permeable chemiluminescent probe used to detect various kinds of ROS. However, it is not only oxidized by ROS but also $ONOO^-$. Luminol is also criticized to undergo redox cycling and may overestimate ROS[144]. To overcome these limitations, we used multiple assays, including DCFH-DA, luminol, and DHE, to assess intracellular ROS and got the consistent results that TFEB inhibits intracellular ROS.

TFEB has been well-established as a master transcription factor regulating lysosomal biogenesis and autophagy. The role of autophagy in inflammation is not fully understood, especially in cardiovascular system[145]. Autophagy protects cells from inflammation-related cell death[146], but also serves an indispensable role in inflammation and immunity against infection[147]. Although we observed an increase of E-selectin, VCAM1, and MCP1 after ATG5 knockdown, compared with the siRNA-control group, siATG5 did not abolish the effect of TFEB on endothelial cell inflammation. This effect is reminiscent of the TFEB inhibition of IL1β secretion induced by LPS and cholesterol crystals independent of Atg5 in macrophages [101]. To further address this dichotomy of effects, we used three autophagy inhibitors, which block autophagy at different steps (3-MA inhibits the initial step of autophagy, bafilomycin and CQ inhibits autophagosome acidification), and got similar results. Although most studies of TFEB focus on autophagy related processes, TFEB has also been found to regulate other types of genes, such as lipid degradation and β-oxidation genes in the liver [64, 148]. Our data here demonstrate that TFEB inhibits endothelial inflammation independent of the canonical autophagy pathway as well, which indicates that TFEB has critical functions beyond acting as an autophagy master gene. Autophagy inhibitors 3-MA, CQ and bafilomycin A1 were used to block autophagy in this study, although there are certain obvious limitations when using these inhibitors. 3-MA is a widely used autophagy inhibitor by inhibiting Class I and Class III phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) [128], although it may have dual roles in the modulation of autophagy, depending on nutritional conditions and treatment time [149, 150] and inhibiting PI3K affects diverse signaling pathways and trafficking process, besides autophagy [151]. CQ is a weak base and inhibits autophagy by impairing lysosomal acidification. In addition to blocking autophagy, CQ also affects other cellular processes such as mitosis and endocytosis [151]. Bafilomycin A1 inhibits the

proton pump on the lysosome membrane thus reducing vesicle acidification [152-154]. However, the function of V-ATPase is also involved in intracellular ions transportation [155], vesicle trafficking [156], proliferation and migration [157]. All these limitations increase the complexity in the a priori interpretation of our data requiring that we use a more specific ATG5-deficient *in vitro* model to demonstrate that impaired autophagy cannot attenuate the anti-inflammatory effect of TFEB. Indeed, we found that bafilomycin A1 treatment enhanced the anti-inflammatory effect of TFEB in the ECs. Perturbation of lysosomal function by bafilomycin A1 promotes TFEB nuclear translocation [158]. In addition, ATP hydrolysis by the v-ATPase is critical to regulate the v-ATPase-Ragulator interaction and promote mTORC1 translocation [153]. Inhibition of lysosome function decreases mTORC1 activity in a feedback loop and induces autophagy [159]. Bafilomycin A1 may decrease mTORC1 activity and possibly induce TFEB nuclear translocation and therefore enhance TFEB anti-inflammatory activity.

In summary, here we demonstrate that TFEB, as an anti-inflammatory factor, negatively regulates leukocyte recruitment and atherosclerosis development. This finding extends our understanding of TFEB in inflammation, oxidative stress, and vascular disease and reveals TFEB as a potential molecular target for treatment of atherosclerosis and associated CVDs.

Chapter 3 TFEB in the vascular smooth muscle cell and aneurysm

Introduction

Aneurysm is a segmental dilatation of the aorta or artery over the normal diameter by 50%, due to the weakness of the vessel wall, leading to over 10,000 deaths every year in the United States. Based on the location of the aneurysm, it can be further divided into abdominal aortic aneurysm (AAA), thoracic aortic aneurysm (TAA) and cerebral aneurysm. Among them, AAA is the most common type of aneurysm[160]. The risk factor of AAA includes older age, male sex, family history, hypertension, elevated cholesterol level, obesity, and preexisting atherosclerotic occlusive disease[161]. An aneurysm can develop over years without any obvious symptoms, making an early diagnosis challenging. Rupture of an aneurysm is often lethal with the mortality over 85%[161]. Current medical intervention for AAA is endovascular repair or repair by surgery. The treatment to control the growth of AAA is to reduce the risk factor with anti-hypertensive drugs or cholesterol-lowering drugs. The drugs targeted at the aneurysm lesion itself is still not available.

The aorta wall composes of different layers. (1) The intima lays the inner face of the aorta. The endothelial cell direct interacts with various cells and signaling molecules in the blood flow; (2) The media layer is the middle layer of the aorta and is mainly made up of smooth muscle cell, elastic fiber, and collagen; (3) The adventitia is the outermost layer of the aorta, providing additional nutritional supporting for the aorta[162]. Vascular smooth muscle cells (VSMCs) are the major residing cell type in the aorta. The contraction of VSMC maintains the

vessel tone and blood pressure[163]. VSMCs also synthesize extracellular matrix protein, such as collagen type I[164] and elastin[165]. VSMCs and the extracellular matrix in the aortic wall withstand the high pressure of the circulating blood in the lumen.

One key feature of the aneurysm is the depletion of vascular smooth muscle cell by apoptosis in the media[166]. Vascular smooth muscle cell apoptosis is prominent in both human and mouse aortic aneurysmal lesion[167, 168]. The loss of VSMC and decreased extracellular protein secretion from VSMCs contribute to the weakness of the aortic wall. Vascular smooth muscle cell apoptosis could be induced by a variety of molecules in the development of the aneurysm, including (1) death-promoting proteins (perforin, Fas, FasL) expressed by infiltrated inflammatory cells[167]; (2) increased oxidative stress from inflammatory cells or VSMC NADPH oxidase; (3) cytotoxic oxidized LDL[169]; (4) loss of survival factors from extracellular matrix[170, 171]. Apoptosis inhibitor shows a promising effect to inhibit the aneurysm development in mouse angiotensin II (Ang II) infusion model[172].

Apoptosis is a programmed cell death process, inherent in all cell types. Apoptosis was first characterized by distinct morphological changes during cell death, including blebbing but intact cytoplasm and condensed nucleus. Mechanistically, apoptosis involves multiple biochemistry processes, including membrane flipping, proteinase activation, and DNA fragmentation. Nevertheless, Caspase family proteins act as the final executor of the apoptosis by the cleavage of multiple target proteins in the cell. Apoptotic cells quickly undergo phagocytosis by macrophages. Apoptosis is crucial to maintaining homeostasis, especially in the case of immune cell development, sexual differentiation, neuron network, and infection[173]. Apoptosis can be initiated by 2 pathways - extrinsic and intrinsic pathway. The extrinsic pathway is mainly through the activation of death receptor on the cell surface. Death receptors belong to the TNFα

receptor superfamily and can be activated with the presence of molecules such as TNFα or Fas ligand (FasL)[174]. The activation of the death receptor forms the death-inducing signaling complex (DISC) with the recruitment of Fas-associated death domain protein (FADD)[175]. DISC then activates the initial Caspase – Caspase 8 in the extrinsic pathway. The alternative way to activate the apoptosis is the intrinsic pathway, in which mitochondria lie at the center of the process. Intrinsic pathway can be initiated by withdrawing of growth factor, DNA damage or chemotherapy. These pathways converge on mitochondrial outer membrane and lead to the release of content from mitochondria into the cytoplasm. Releases of Cytochrome c from mitochondria activates Caspase 9/Apaf-1 complex and triggers downstream apoptotic steps[176].

Here, we found that TFEB mRNA and protein abundance is reduced in the human aneurysmal lesion. TFEB inhibits VSMC apoptosis by promoting BCL2 transcription. Smooth muscle cell (SMC) specific *Tfeb* knockout (KO) promotes VSMC apoptosis and aortic aneurysm development in both proprotein convertase subtilisin/kexin type 9 (PCSK9) / Angiotensin II (AngII)[177] and β-aminopropionitrile (BAPN) / AngII [178] mouse aneurysm model. Administration of (2-Hydroxypropyl)-β-cyclodextrin (HPβCD), a TFEB activator, prevents mouse aneurysm in PCSK9/AngII model.

Materials and methods

Materials and reagents

Antibodies against TFEB, β-actin, PARP, Caspase3 were from Cell Signaling Technology (CST, Danvers, MA). BCL2 antibody was from Abcam (UK). Flag antibody, alexidine dihydrochloride, and HPβCD were Sigma-Aldrich (St. Louis, MO). Recombinant human TNFα, IL1β, IFNγ, and pan-caspase inhibitor Z-VAD-FMK were from R&D systems (Minneapolis, MN). Recombinant Fas Ligand was from Enzo Life Science (Ann Arbor, MI).

Chloroquine, 3MA were from Sigma-Aldrich (St. Louis, MO). Bafilomycin A1, cycloheximide, ABT199 and HA14-1 were from Cayman Chemical (Ann Arbor, MI). Human aorta and aneurysm samples were from cardiac surgery department of the University of Michigan hospital.

Cell culture

Human aortic smooth muscle cells (HASMCs) were purchased from Lonza. HCAECs were cultured in Smooth Muscle Cell Growth Medium 2 (Promo Cell, Germany) at 37°C/5% CO2 humidified incubator. CV-1 cells for the luciferase assay was purchased from ATCC and cultured in DMEM with 10% FBS (Gibco).

Animal procedures

C57BL/6N-A^{tm1Brd}/a Tfeb$^{tm1a(EUCOMM)Wtsi}$/BcmMmucd mice were produced at BCM from ES cells provided by the Wellcome Trust Sanger institute within the NIH funded KOMP2 project. The *Tfeb*^{flox} mice contain loxP sites flanking exons 4 and 5 of Tfeb[179]. Myh11-creER^{T2} mouse was purchased from Jackson Laboratory (Jackson Laboratory Stock No: 019079, Bar Harbor, ME). The cre was driven by mouse smooth muscle myosin, heavy polypeptide 11, smooth muscle (Myh11) promoter/enhancer regions on the BAC transgene and used for deletion of genes in smooth muscle cells[180]. The cre transgene is on the Y chromosome, so only male mice were used for this study. *Tfeb*^{flox} mice were crossbreeding Myh11-creER^{T2} to generate SMC specific *Tfeb* KO mice (*Tfeb*$^{\Delta SMC}$). *Tfeb*^{flox} and *Tfeb*$^{\Delta SMC}$ mice were injected with tamoxifen (T5648, Sigma-Aldrich) 2mg for continuously 5 days intraperitoneally and wait for 9 days before the experiment.

The PCSK9/AngII model was performed according to previously publication[177]. Ten- to 12-week-old mice were injected with Adeno-associated Virus (AAV, serotype 8) containing PCSK9D377Y gain-of-function mutation with the dosage 2* 10^{11} genome copy per mouse and

fed with high-cholesterol diet (HCD, 17.3% protein, 21.2% fat, 48.5%carbohydrate, 0.2%cholesterol by mass, and 42% calories from fat; TD.88137, Envigo) to induce hyperlipidemia. After 2 weeks, Angiotensin II (1,000 or 1500 ng/kg/min) (H1706, Bachem) was infused subcutaneously via mini-pump (Alzet, model 2004) for 4 weeks. Mice were sacrificed 4 weeks after the surgery. The diameter of the aorta was measured with a digital caliper in a double-blind way. Aneurysm was defined as a dilation of aorta greater than 50% of the normal aorta (greater than 1.2mm).

The BAPN/Ang II model was performed according to the previous publication[178]. Ten- to 12-week-old mice were infused with AngII (1000ng/kg/min) for 4 weeks with mini-pump (Alzet, model 2004). BAPN (150mg/kg/day) was infused subcutaneously for the first 2 weeks with mini-pump (Alzet, model 2002). Mice were sacrificed 4 weeks after the surgery.

HPβCD (H107, Sigma-Aldrich) intraperitoneally injection (2g/kg) was performed twice a week during AngII infusion. HPβCD was dissolved in saline freshly before injection.

Blood samples were sent to the Chemistry Laboratory of the Michigan Diabetes Research and Training Center to determine lipid profile All animal work was performed in accordance with the University of Michigan Animal Care and Use Committee.

Annexin V apoptosis assay

HASMCs were treated with TNFα (100ng/mL) + CHX (20μM) for 4 hours in Opti-MEM medium (31985062, Gibco). Cells were disassociated with 0.25% trypsin (25200056, Gibco). The cells were stained with FITC Annexin V Apoptosis Detection Kit I (556548, BD Biosciences). In brief, cells were washed with cold PBS, suspended in 1* binding buffer and stained with FITC Annexin V and Propidium Iodide (PI). The flow cytometry was performed in flow cytometry core of the University of Michigan.

TUNEL apoptosis assay

Paraffin-Embedded Tissue sections of aorta were stained with ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100, Millipore Sigma) and counterstained with 0.5% methyl green.

RNA preparation and RT-qPCR analysis

Total RNA was extracted from cells using RNeasy Kit (QIAGEN, Hilden, Germany), followed by reverse transcription with SuperScript III kit (Invitrogen, Carlsbad, CA) and random primers. mRNA was determined by qPCR (BioRad, Hercules, CA), using iQ SYBR Green Supermix (BioRad, Hercules, CA). The mRNA level was normalized to internal control - ACTB unless otherwise mentioned. The primers used are shown in Table 3.1.

Gene*	Primer sequence
hTFEB	Forward: GCGGCAGAAGAAAGACAATC
	Reverse: CTGCATCCTCCGGATGTAAT
hBCL2	Forward: TCATGTGTGTGGAGAGCGTC
	Reverse: GCCGTACAGTTCCACAAAGG
hBCL2A1	Forward: AGTGCTACAAAATGTTGCGTTC
	Reverse: GGCAATTTGCTGTCGTAGAAGTT
hBAD	Forward: CCCAGAGTTTGAGCCGAGTG
	Reverse: CCCATCCCTTCGTCGTCCT
hBAX	Forward: CCCGAGAGGTCTTTTTCCGAG
	Reverse: CCAGCCCATGATGGTTCTGAT
hBCLx	Forward: GACTGAATCGGAGATGGAGACC
	Reverse: GCAGTTCAAACTCGTCGCCT
hBAK1	Forward: CATCAACCGACGCTATGACTC
	Reverse: GTCAGGCCATGCTGGTAGAC
hBIM	Forward: CCAGGCCTTCAACCACTATC
	Reverse: CCCTCCTTGCATAGTAAGCG
hBID	Forward: ATGGACCGTAGCATCCCTCC
	Reverse: GTAGGTGCGTAGGTTCTGGT
hP53	Forward: GAGGTTGGCTCTGACTGTACC
	Reverse: TCCGTCCCAGTAGATTACCAC

mTFEB	Forward: GGGCTACATCAACCCTGAGA
	Reverse: CTGCATCCTCCGGATGTAAT
mBCL2	Forward: ATGCCTTTGTGGAACTATATGGC
	Reverse: GGTATGCACCCAGAGTGATGC
hBCL2 ChIP	Forward: CCTCTCCCCTGTCTCTCTCC
	Reverse: CCCTTCTCGGCAATTTACAC

Table 3.1 Primers used for Real-time PCR. h=human m=mouse

Lactate Dehydrogenase (LDH) Activity Assay

LDH assay was performed with the LDH kit (MAK066) from Sigma-Aldrich following the manufacturer's protocol.

Protein extraction and Western blot

Cells were lysed in RIPA lysis buffer (Thermo Scientific, Waltham, MA) with a protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Proteins were resolved in 10% SDS-PAGE gel and transferred to nitrocellulose membrane (BioRad, Hercules, CA). Membranes were blocked for 1 hour at room temperature in TBST containing 5% fat-free milk and incubated with primary antibody (1:1000) at 4 °C overnight. After TBST washing, membranes were incubated with secondary antibody (Li-Cor bioscience, Lincoln, NE) (1:8000) for 1 hour at room temperature. After TBST washing, bands were analyzed using an image-processing program (Li-Cor Odyssey).

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed with SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (#9003, CST), according to manufacturer's protocol. Purified precipitated DNA was used as the template for qPCR and primers used were listed in Supplemental Table 3.1.

siRNA-mediated gene knockdown

siTFEB (AGACGAAGGUUCAACAUCA)) was from (Dharmacon, Lafayette, CO). siAtg7 (GCUCGCUUAACAUUGGAGUtt) was from Ambion (Austin, TA). Endothelial cells were transfected with siRNA or non-targeting siRNA (Ambion In Vivo Negative Control #1 siRNA, Thermo Scientific, Waltham, MA) (30nM) using Lipofectamine RNAiMAX Reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol.

Plasmid construction and transfection

Desired DNA fragments of BCL2 promoter (-547 - +16) from human genome were PCR-amplified and cloned into pGL4.11 luciferases reporter vector (Promega, Madison, WI). Mutation of the putative binding site was performed using Q5 Site-Directed Mutagenesis Kit (New England Biolabs, Ipswich, MA). All PCR products were verified by DNA sequencing. CV1 cells were transfected with luciferase plasmid at 70-80% confluence, using lipofectamine 2000 (Invitrogen, Carlsbad, CA) in accordance with the suggested protocol. Promoter activity was detected by Firefly luciferase and normalized against Renilla luciferase activity.

Construction of adenoviruses

Adenoviruses encoding GFP and human TFEB were generated by cloning the coding region of human TFEB and control GFP into AdTrack-CMV (Aglient Technologies, Santa Clara, CA). Next, the coding region was cloned from Ad-track into Ad-Easy vector by homologous recombination in *Escherichia coli*. The adenovirus encoding LacZ and human TFEB were generated by cloning the coding region of TFEB and control LacZ into PCR8/GW/TOPO TA vector (Invitrogen, Carlsbad, CA). The adenovirus encoding flag-TFEB was generated by inserting flag tag to the N-terminal of human TFEB coding region. Next, the sequence was cloned from Entry Vector to the pAd/CMV/V5-DEST Vector (Invitrogen, Carlsbad, CA) by LR recombination. The adenoviruses were packaged in HEK293 cells and purified by CsCl2 density gradient ultracentrifugation. Adenovirus titration was determined by the Adeno-XTM quantitative PCR titration kit (Clontech, CA, USA).

Statistics

Data are presented as mean ± SEM. Student t test or 1-way ANOVA followed by Holm-Sidak test was used to analyze data. A $p<0.05$ was considered as statistical significance. All results were represented from at least 3 independent experiments.

Results

TFEB is reduced in human aneurysmal lesion

To address if TFEB expression is altered in the human aneurysm, we took the aortic tissue removed in the aorta repair surgery from the patient with the aneurysm. We compare the TFEB expression in the aneurysmal lesion, with the adjacent non-lesion site. Immunofluorescence indicated that TFEB is decreased in the media layer of the aneurysmal lesion (Fig. 3.1 A). After the removal of adventitia, we also determined that both TFEB mRNA and protein abundance is reduced in the aneurysmal lesion (Fig. 3.1 B-D). Consistent with the *in vivo* data, pro-inflammatory cytokines (TNFα, IL1β or IFNγ) also reduces TFEB protein in the human aortic smooth muscle cells (HASMCs) (Fig. 3.1 E-F).

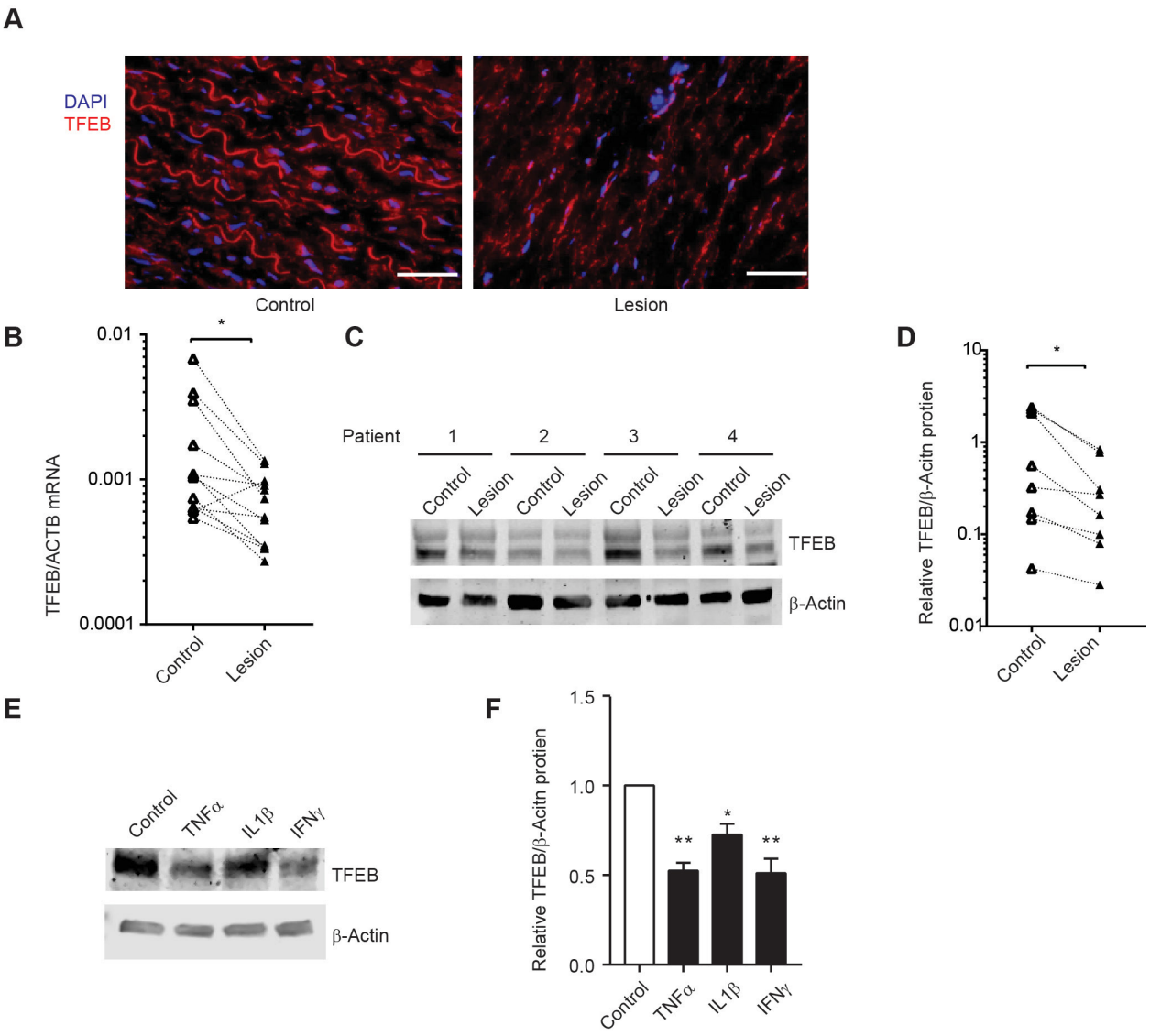

Figure 3.1 TFEB is reduced in human aneurysmal lesion.

(A) TFEB protein was determined by immunofluorescence in human aneurysmal lesion, compared with adjacent normal site. (B) TFEB mRNA was determined by qPCR in human aneurysmal lesion, compared with adjacent normal site. (C and D) TFEB protein was determined by Western blot in human aneurysmal lesion, compared with adjacent normal site. (E and F) HASMCs were treated with TNFα (20ng/mL), IL1β (10ng/mL) or IFNγ (50ng/mL) for 72 hours. TFEB protein was determined by Western blot. * $p<0.05$ ** $p<0.01$ Data are presented as mean ± SEM.

TFEB inhibits HASMC apoptosis

Apoptosis of VSMCs in the aorta media is critical for the weakness of aortic wall and the subsequent development of an aneurysm. To determine if TFEB affects HASMC apoptosis, we used multiple assays to assess apoptosis *in vitro*. TFEB knockdown promotes HASMC death induced by either Fas ligand (FasL)[181] or TNFα+cycloheximide (CHX)[182], assessed by lactate dehydrogenase (LDH) released into the medium. The cell death can be blocked by pan-caspase inhibitor - carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (zvad), indicating it is an apoptosis-dependent process (Fig. 3.2 A). To specifically investigate the apoptosis, we used Western blot to probe for the apoptosis markers – the cleavage of Poly (ADP-ribose) polymerase (PARP)[183] and cysteine-aspartic proteases, cysteine aspartases 3 (Caspase3)[184]. TFEB overexpression by adenovirus in HASMC significantly decreases the cleavage of both PARP and Caspase3 (Fig. 3.2 B-C). Consistently, TFEB knockdown in HASMC significantly increases the cleavage of both PARP and Caspase3 (Fig. 3.2 D-E). The exposure of phosphatidylserine at the outer leaflet of the plasma membrane is an early marker of apoptosis. Annexin V can bind to the exposed PS phosphatidylserine of the apoptotic cells. Combined with propidium iodide (PI) staining, this assay can distinguish apoptotic cells (Annexin V+/PI-) from necrotic cells (Annexin V+/PI+). By performing Annexin V assay in both TFEB overexpressed or knockdown cells, we confirmed that TFEB overexpression reduces (17.03% to 11.2%) (Fig. 3.2 F), while TFEB knockdown increase (10.77% to 15.73%) (Fig. 3.2 G), apoptotic cell population, induced by TNFα+CHX.

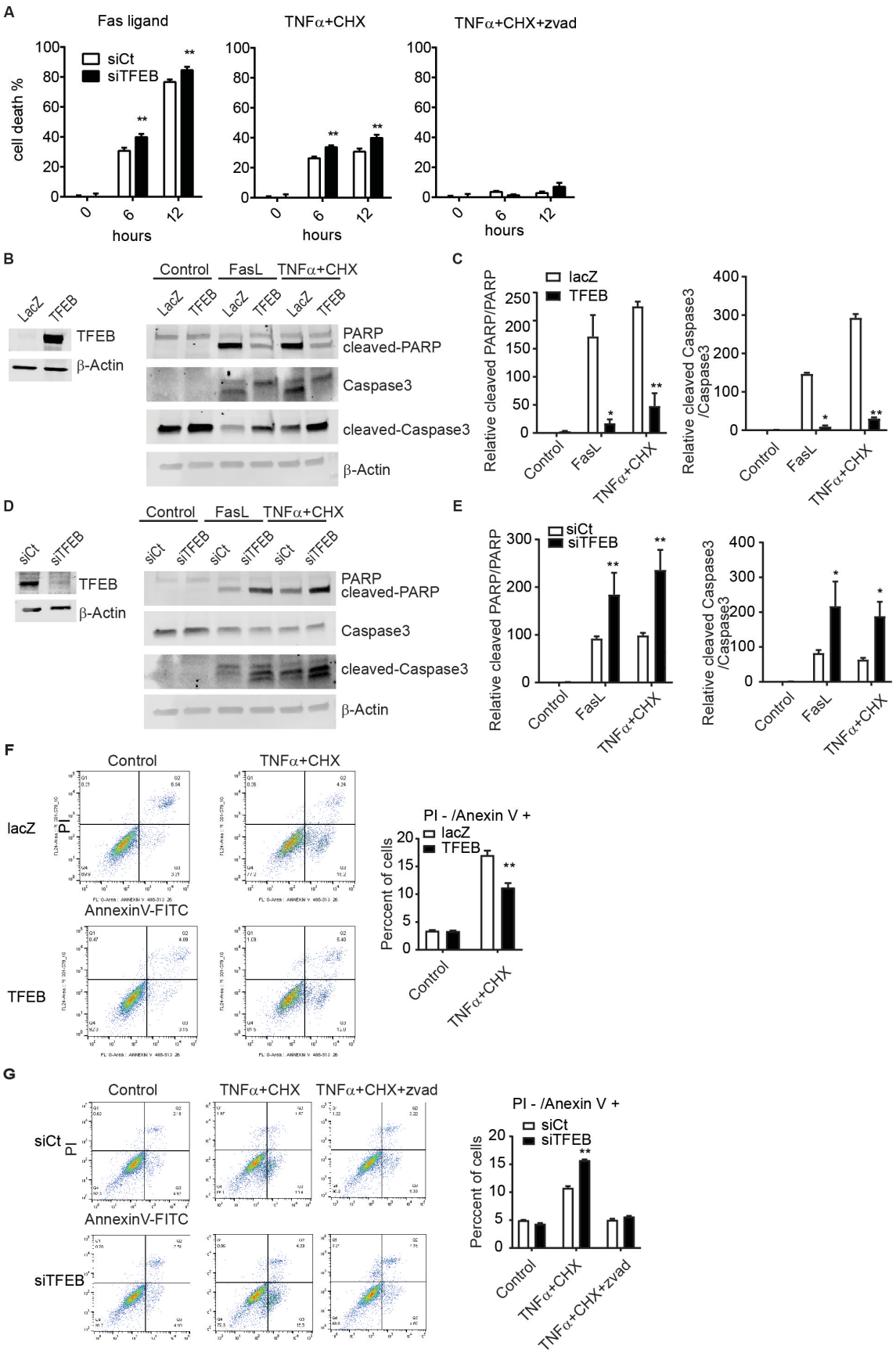
A
Fas ligand
TNFα+CHX
TNFα+CHX+zvad
siCt
siTFEB
cell death %
hours
B
Control
FasL
TNFα+CHX
LacZ
TFEB
β-Actin
PARP
cleaved-PARP
Caspase3
cleaved-Caspase3
C
lacZ
TFEB
Relative cleaved PARP/PARP
Relative cleaved Caspase3 /Caspase3
D
siCt
siTFEB
E
F
Control
TNFα+CHX
lacZ
TFEB
PI
AnnexinV-FITC
PI - /Anexin V +
Perccent of cells
G
TNFα+CHX+zvad

Figure 3.2 TFEB inhibits HASMC apoptosis.

(A) HASMCs were treated with FasL (100ng/mL) or TNFα (100ng/mL) + CHX (20μg/mL) for indicated time and LDH activity in the medium was measured respectively. (B and C) HASMCs were infected with adenovirus encoding LacZ (Ad-lacZ) or TFEB (Ad-TFEB) (30MOI). After 48 hours, cells were treated FasL or TNFα+CHX for 6 hours before protein was harvest for Western blot. (D and E) HASMCs were transfected with small interfering (siRNA) negative control (siCt) or siRNA against TFEB (siTFEB). After 72 hours, cells were treated FasL or TNFα+CHX for 6 hours before protein was harvest for Western blot. (F) HASMCs were infected with Ad-lacZ or Ad-TFEB (30MOI) for 48 hours and then treated with TNFα+CHX for 4 hours before Annexin V and PI staining. (G) HASMCs were transfected with siCt or siTFEB for 72 hours and then treated with TNFα+CHX for 4 hours before Annexin V and PI staining. * $p<0.05$ **$p<0.01$ Data are presented as mean ± SEM of three independent experiments.

TFEB inhibits HASMC apoptosis via upregulation of BCL2

To investigate the possible pathways mediating the anti-apoptotic effect of TFEB in HASMCs, we assess the important anti-apoptotic BCL2 family and pro-apoptotic P53 mRNA in HASMCs after TFEB overexpression and knockdown (Fig. 3.3 A-B). Among them, we found that BCL2, an important anti-apoptotic protein on the mitochondria membrane, is increased by TFEB overexpression and decreases by TFEB knockdown. This is further confirmed by Western blot (Fig. 3.3 C-D). Since TFEB is a transcription factor, we performed chromatin immunoprecipitation (ChIP) assay and found that TFEB directly binds to the putative E-box in the promoter of *BCL2* (Fig 3.3 E). TFEB also increases *BCL2* promoter driving luciferases activity and mutation of the putative binding site diminish this effect (Fig 3.3 F), further confirming the direct binding of TFEB. Importantly, pretreated the cells with BCL2 inhibitor ABT199[185] or HA14-1[186] abolishes the anti-apoptotic effect of TFEB in HASMC induced by TNFα + CHX, indicating an indispensable role of BCL2 as a downstream pathway of TFEB (Fig. 3.3 G-H).

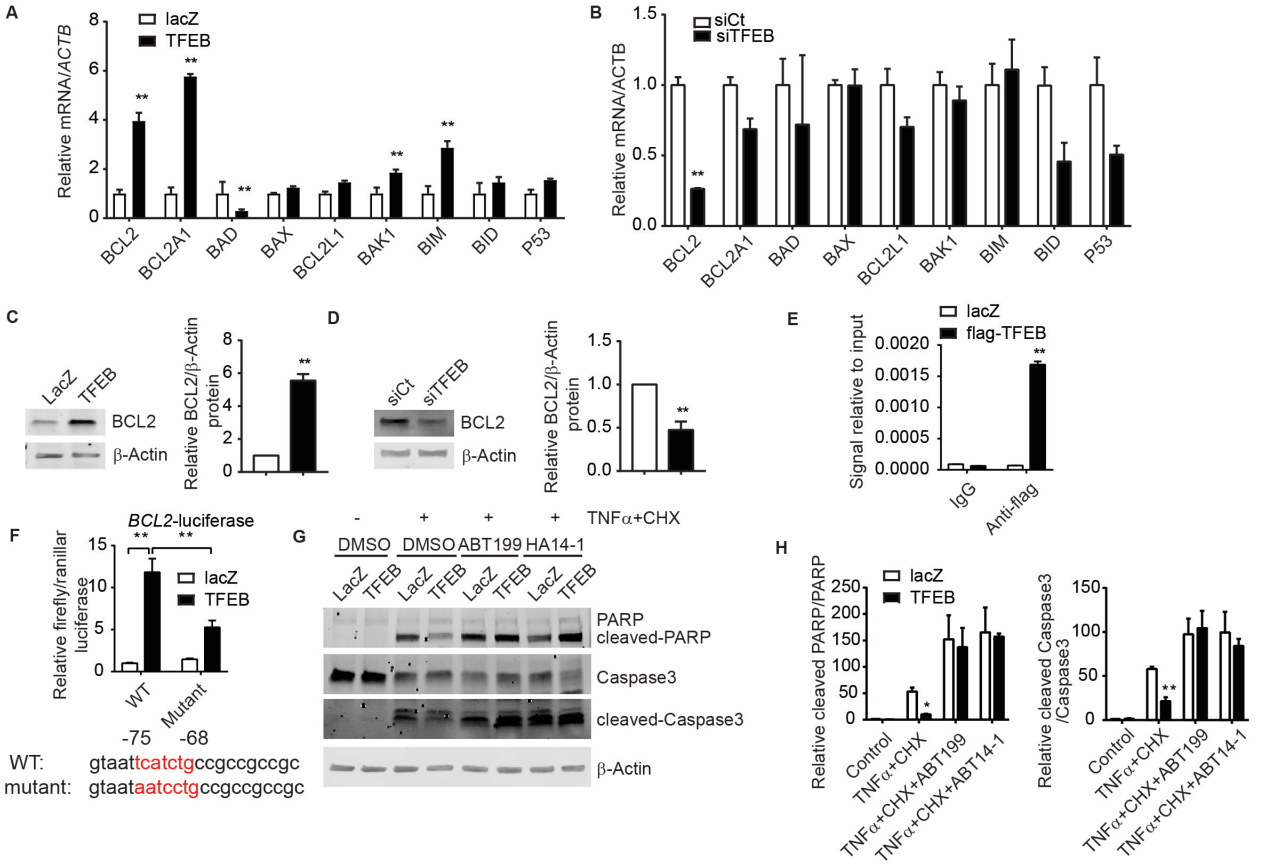

Figure 3.3 TFEB inhibits HASMC apoptosis via upregulation of BCL2.

(A and C) HASMCs were infected with Ad-lacZ or Ad-TFEB (30MOI) for 48 hours and RNA or protein was harvest for qPCR. (B and D) HASMCs were transfected with siRNA negative control or against TFEB for 72 hours and RNA was harvest for qPCR. (E) HASMCs were infected with Ad-lacZ and Ad-flag-TFEB for 48 hours. ChIP were performed using antibodies against flag or normal rabbit IgG. The binding of TFEB to *BCL2* promoter was determined by qPCR. (F) CV1 cells were transfected with *BCL2* promoter driving luciferase in PGL4 vector. After 24 hours, cells were infected with Ad-lacZ and ad-TFEB for 48 hours and the luciferase activity was determined by luminometers. (G-H) HASMCs were infected with Ad-lacZ and Ad-TFEB (30MOI) for 48hours. After that, cells were pretreated with ABT199 (200nM) or HA14-1 (20μM) for 1 hour and then treated with TNFα+CHX for 6 hours before protein was harvested for Western blot. * $p<0.05$ **$p<0.01$ Data are presented as mean ± SEM of three independent experiments.

TFEB inhibits apoptosis independent of autophagy

TFEB has emerged as a master regulator of lysosomal biogenesis and autophagy. To determine if the autophagy pathway is related to the anti-apoptotic effect of TFEB in HASMC, we used autophagy inhibitor - Bafilomycin A1 (BFA) and 3-Methyladenine (3MA). Pretreat HASMC with BFA(200nM) or 3-Methyladenine (3MA) (5mM) for 12 hours before adding apoptosis inducer cannot abolish the effect of TFEB on cleavage of Caspase3 and PARP (Fig. 3.4A). In addition to pharmacological inhibition, we also used siRNA against ATG7[187], an essential protein for autophagy, to inhibit the autophagy in HASMCs. Knockdown of ATG7 cannot abolish the anti-apoptotic effect of TFEB in HASMCs either (Fig. 3.4B). As a result, TFEB inhibits apoptosis independent of autophagy.

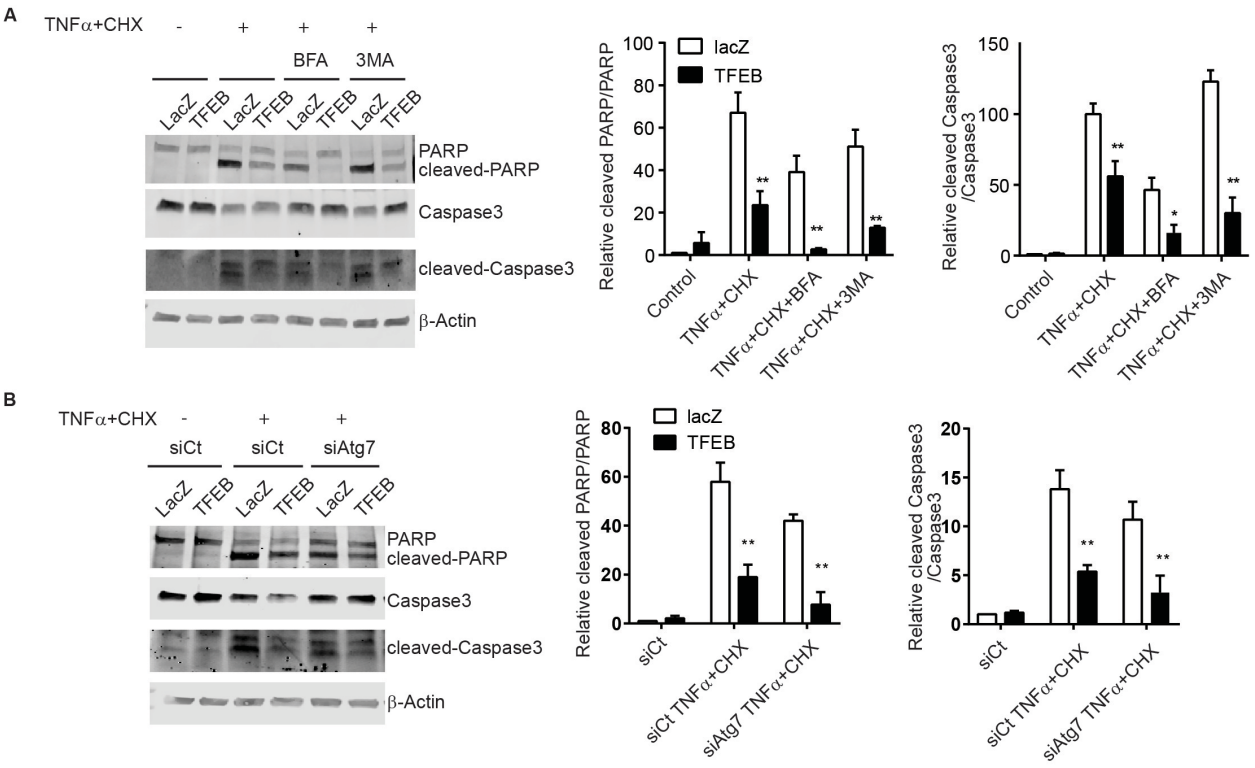

Figure 3.4 TFEB inhibits apoptosis independent of autophagy.

(A) HASMCs were infected with Ad-lacZ and Ad-TFEB (30MOI) for 36 hours. After that, cells were pretreated with bafilomycin A1 (BFA) (200nM) or 3-Methyladenine (3MA) (5mM) for 12 hours and then treated with TNFα+CHX for 6 hours before protein was harvested for Western blot. (B) HASMCs were transfected with siRNA and siAtg7 for 12 hours and then infected with Ad-lacZ or Ad-TFEB (30MOI) for 48 hours. After that, cells were treated with TNFα+CHX for 6 hours before protein was harvested for Western blot. * $p<0.05$ **$p<0.01$ Data are presented as mean ± SEM of three independent experiments.

Characterization of SMC specific Tfeb KO mice.

To determine the role of SMC TFEB *in vivo*, we generated SMC specific *Tfeb* KO mouse (*Tfeb*$^{\Delta SMC}$) by crossbreeding *Tfeb*^{flox} mice with *Myh11*-cre/ER^{T2} mice (Fig. 3.5 A). The knockout efficiency was determined by both qPCR and Western blot (Fig. 3.5 B-D). Consistent with the *in vitro* data, knockout *Tfeb in vivo* also significantly reduces BCL2 mRNA and protein abundance in the aorta. To exclude the possible compensation of other MIT/TFE family genes, MITF and TFE3 protein in the aorta from *Tfeb*^{flox} and *Tfeb*$^{\Delta SMC}$ were determined by Western blot and we did not find a significant change of either gene. (Fig. 3.5 E-F).

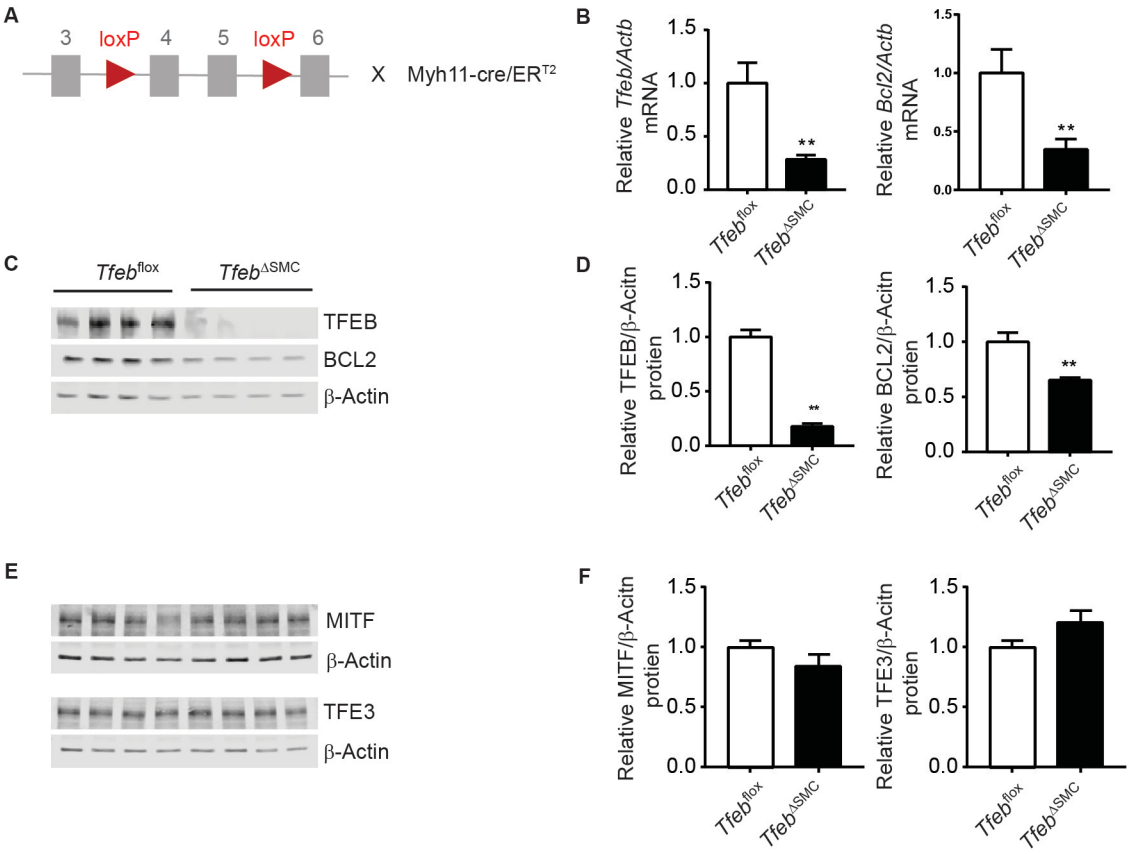

Figure 3.5 Characterization of SMC specific *Tfeb* KO mice.

(A) Schematics of SMC specific *Tfeb* KO mice (*Tfeb*$^{\Delta SMC}$). (B-F) After adventitia was removed, the aorta was lysed for qPCR (B) and Western blot (C-F). * $p<0.05$ **$p<0.01$ Data are presented as mean ± SEM.

Tfeb KO promotes aneurysm development and VSMC apoptosis in PCSK9/Ang II model.

To determine the role of TFEB in VSMC *in vivo*, we induced aneurysm in both *Tfeb*^{flox} and *Tfeb*$^{\Delta SMC}$ mice by the AAV-PCSK9 injection and Ang II infusion (Fig. 3.6 A). The mice were euthanized at day 28 and the diameter of the abdominal aorta was measured. *Tfeb* KO in SMC significantly increases both the maximal diameter of the abdominal aorta (1.182 to 1.51mm) and the incidence of the AAA (40.9% to 64%) (Fig. 3.6 B and D). Blood pressure was comparable between *Tfeb*^{flox} and *Tfeb*$^{\Delta SMC}$ (Fig. 3.6 C). We also assessed the VSMC apoptosis in the aorta by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Consistent with the *in vitro* data, *Tfeb* KO in SMC also significantly increases the apoptotic cell numbers in the aortic media (Fig 3.6 E).

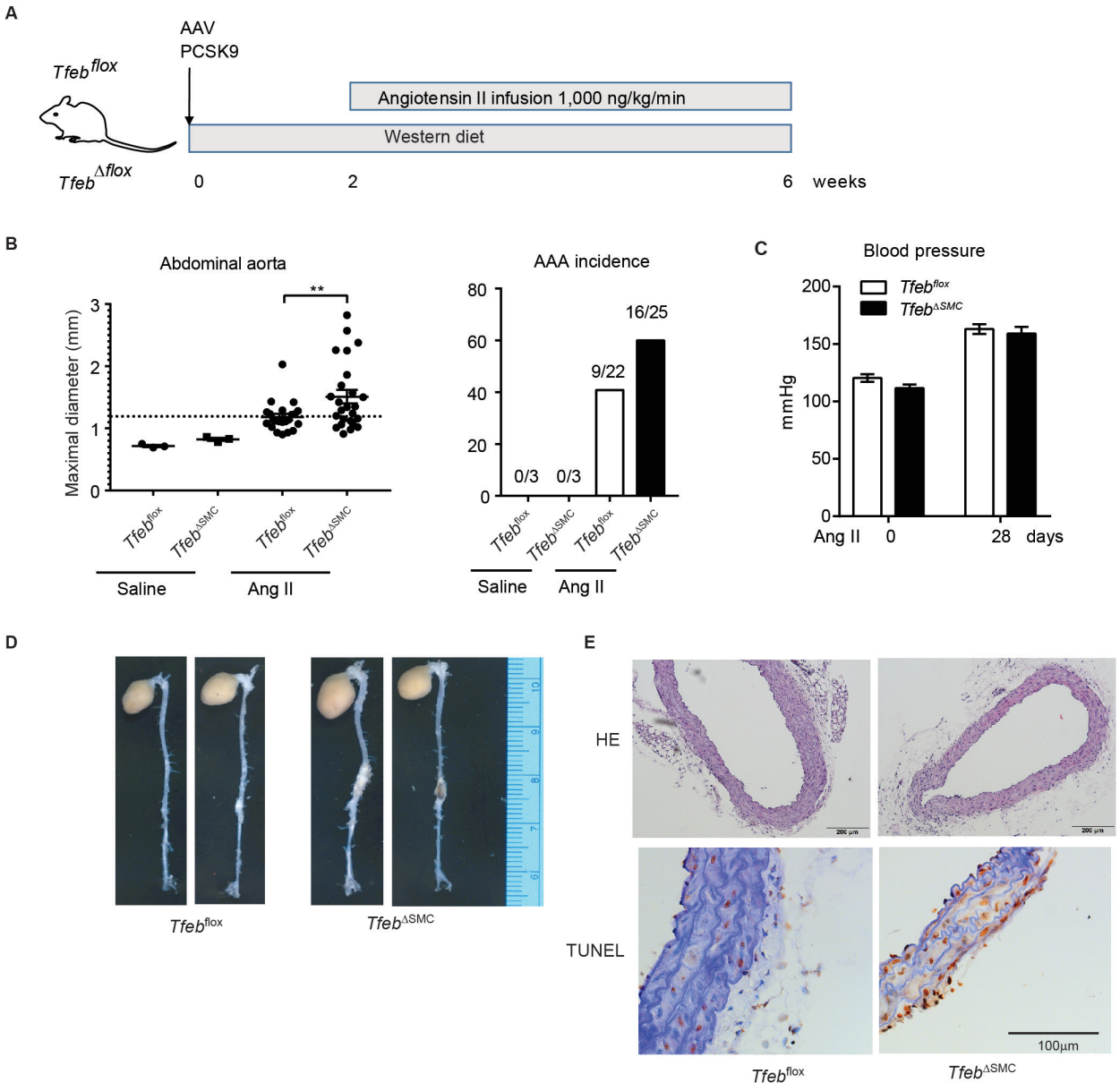

Figure 3.6 *Tfeb* KO promotes aneurysm development and VSMC apoptosis in PCSK9/Ang II model.

(A) Schematics of the PCSK9/Ang II model. (B) Maximal diameter and abdominal aneurysm incidence were determined at day 28. (C) Blood pressure was measured on day 0 and day 28 of angiotensin II infusion. (D) Representative photo of the aortas from 2 groups. (E) Representative section of the abdominal aorta from 2 groups (HE staining and TUNEL). * $p<0.05$ ** $p<0.01$ Data are presented as mean ± SEM. n=3 for saline treatment n=22 for Ang II treatment.

Tfeb KO promotes aneurysm development and VSMC apoptosis in BAPN/Ang II model.

We also applied another murine aneurysm model to investigate the role of TFEB in VSMC and aneurysm development, in which aneurysm is induced by β-aminopropionitrile (BAPN) and Ang II. (Fig. 3.7 A). Because of the high incidence and mortality rate of this model, we found that *Tfeb* SMC KO significantly increases the rupture and reduces the survival rate of the mice (66.67% to 16.67%) (Fig. 3.7 B-C). *Tfeb* KO in SMC significantly increases both the maximal diameter of both the thoracic (1.632 to 1.862mm) and abdominal aorta (1.182 to 1.51mm) (Fig. 3.7 D and F) without affecting the blood pressure (Fig 3.7 E). TUNEL assay also demonstrated the increase of VSMC apoptosis in *Tfeb*$^{\Delta SMC}$ mouse aorta. (Fig 3.7 G).

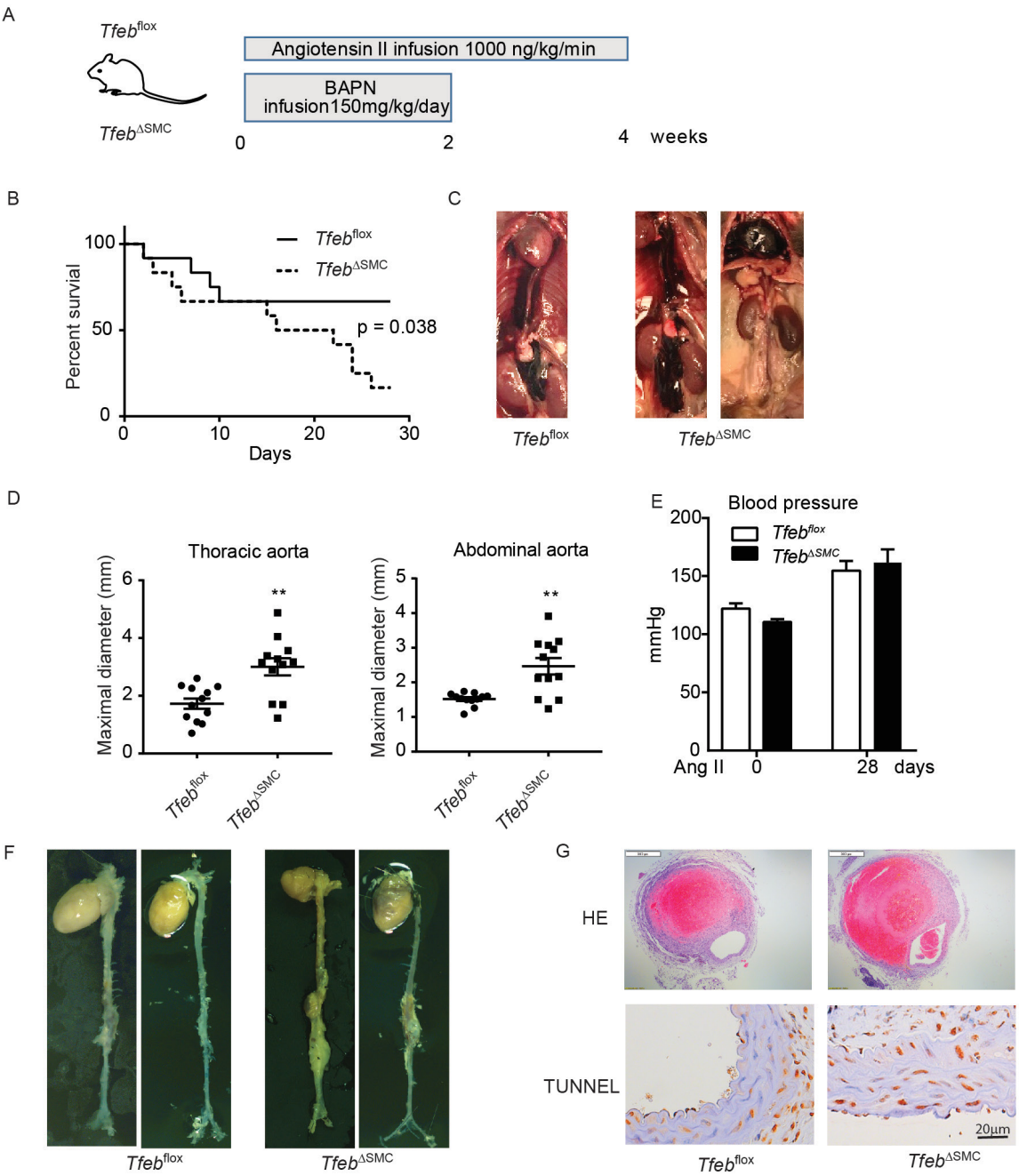

Figure 3.7 *Tfeb* KO promotes aneurysm development and VSMC apoptosis in BAPN/Ang II model.

(A) Schematics of the BAPN/Ang II model. (B) Survival curve of *Tfeb*^{flox} and *Tfeb*$^{\Delta SMC}$ mice. (C) Representative photo of the ruptured aorta. (D) Blood pressure was measured on day 0 and day 28 of angiotensin II infusion. (E) Maximal diameter of thoracic and abdominal aorta were determined at day 28. (F) Representative photo of the aortas from 2 groups. (G) Representative section of the abdominal aorta from 2 groups (HE staining and TUNEL). * $p<0.05$ **$p<0.01$ Data are presented as mean ± SEM. n= 12 for each genotype.

HPβCD activates TFEB and attenuates aneurysm development

There has been numerous of TFEB activators has been published as discusses in Chapter 1. We test alexidine dihydrochloride (AD) [188], digoxin[188], 2-hydroxypropyl-β-cyclodextrin (HPβCD)[189], and naringenin[85]. They all significantly increase the nuclear translocation of the TFEB-EGFP fusion protein in HASMCs (Fig. 3.8 A). To further explore the pharmaceutical potential of TFEB activator in the aneurysm, we performed the injection of AD and HPβCD in the PSCK9/Ang II aneurysm model (twice per week) (Fig. 3.8 B). After 28days, we found that HPβCD, but not AD, reduces the maximal abdominal aorta diameter and abdominal aorta aneurysm incidence (Fig. 3.8 C). Either of the drug influences mouse body weight, plasma total cholesterol or triglycerides, indicating a possible localized effect of the drug (Fig. 3.8 D). To confirm if HPβCD indeed attenuates aneurysm in a TFEB dependent manner, we performed the injection and aneurysm model in both *Tfeb*^{flox} and *Tfeb*$^{\Delta SMC}$ mice (Fig. 3.8 E). HPβCD attenuates aneurysm development in *Tfeb*^{flox} but not *Tfeb*$^{\Delta SMC}$ mice, indicating the pharmaceutical effect of HPβCD requires SMC TFEB (Fig. 3.8 F).

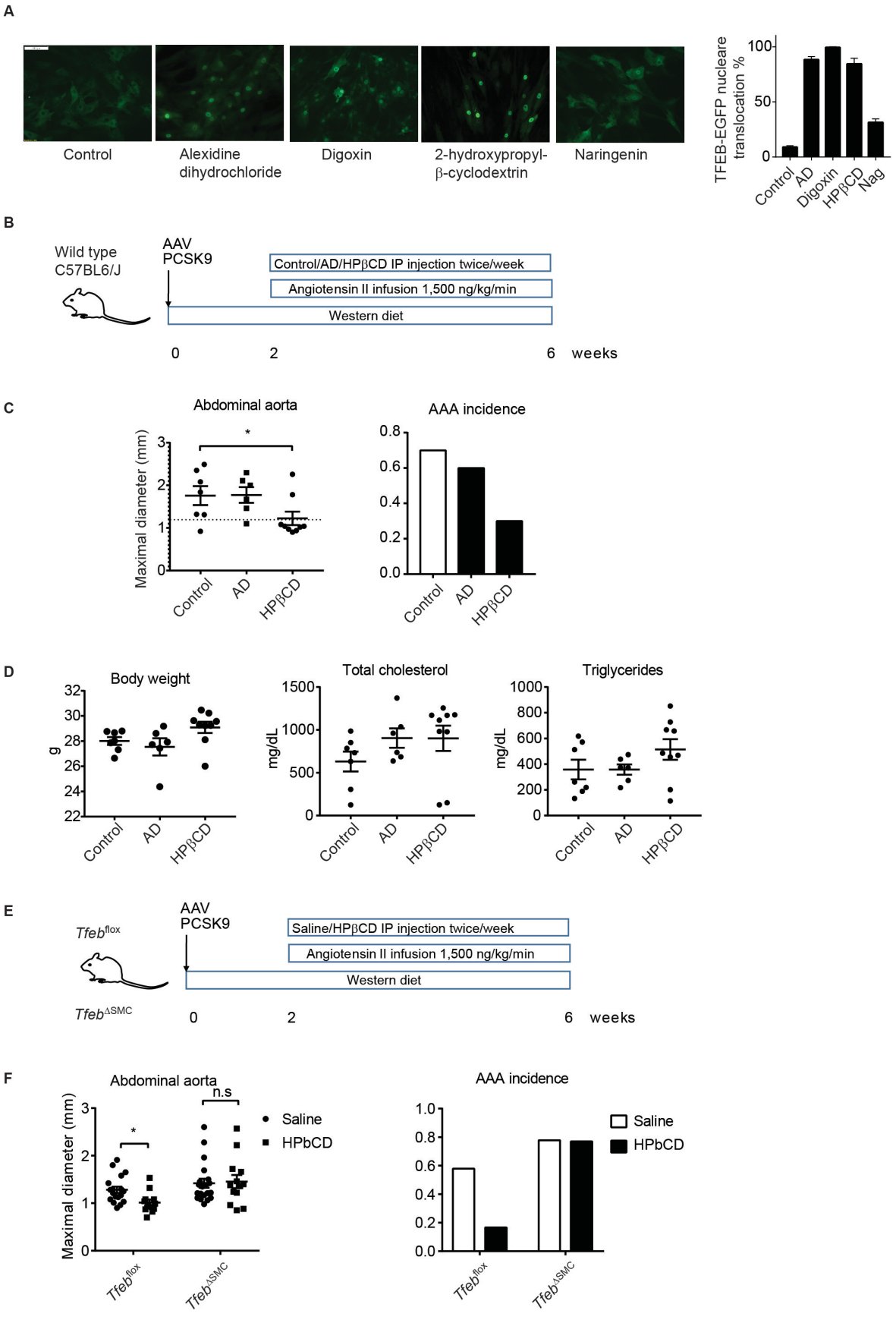
A
Control
Alexidine dihydrochloride
Digoxin
2-hydroxypropyl-β-cyclodextrin
Naringenin
TFEB-EGFP nucleare translocation %
100
50
0
Control
AD
Digoxin
HPβCD
Nag
B
Wild type C57BL6/J
AAV PCSK9
Control/AD/HPβCD IP injection twice/week
Angiotensin II infusion 1,500 ng/kg/min
Western diet
0
2
6 weeks
C
Abdominal aorta
Maximal diameter (mm)
*
Control
AD
HPβCD
AAA incidence
D
Body weight
g
Total cholesterol
mg/dL
Triglycerides
mg/dL
E
Tfeb^flox
Tfeb^ΔSMC
AAV PCSK9
Saline/HPβCD IP injection twice/week
Angiotensin II infusion 1,500 ng/kg/min
Western diet
0
2
6 weeks
F
Abdominal aorta
n.s
Saline
HPbCD
Maximal diameter (mm)
AAA incidence

Figure 3.8 HPβCD activates TFEB and attenuates aneurysm development.

(A) HASMCs were infected with adenovirus encoding TFEB-EGFP fusion protein (20MOI) for 24 hours and treated with the indicated drug for 6 hours. (B) Schematics of the drug injection and aneurysm model. (C) Maximal diameter of abdominal aorta and abdominal aorta aneurysm (AAA) incidence were determined at day 28. (D) Body weight and plasma lipid profile were determined at day 28. (E) Schematics of the drug injection and aneurysm model. (F) Maximal diameter of abdominal aorta were and abdominal aorta aneurysm (AAA) incidence were determined at day 28. * $p<0.05$ **$p<0.01$ Data are presented as mean ± SEM.

HPβCD inhibits HASMC apoptosis dependent of TFEB

HASMCs were pretreated with different dosage of HPβCD (6, 8, 10 mg/mL) before adding apoptosis inducer and the result indicating HPβCD inhibits apoptosis in a dosage-dependent manner (Fig 3.9 A). In addition, TFEB knockdown by siRNA abolishes the anti-apoptotic effect of HPβCD in HASMCs (Fig 3.9 B). From the aforementioned study, BCL2 is a target of TFEB in HASMCs. Consistently, HPβCD increase both BCL2 mRNA and protein in HASMCs in a TFEB dependent manner (Fig 3.9 C). Furthermore, HPβCD also upregulates other well-known TFEB target genes, such as GLA, MAP1LC3B, and SQSTM1 as well as the turnover of autophagy markers LC3 in a TFEB-dependent way (Fig. 3.9 D).

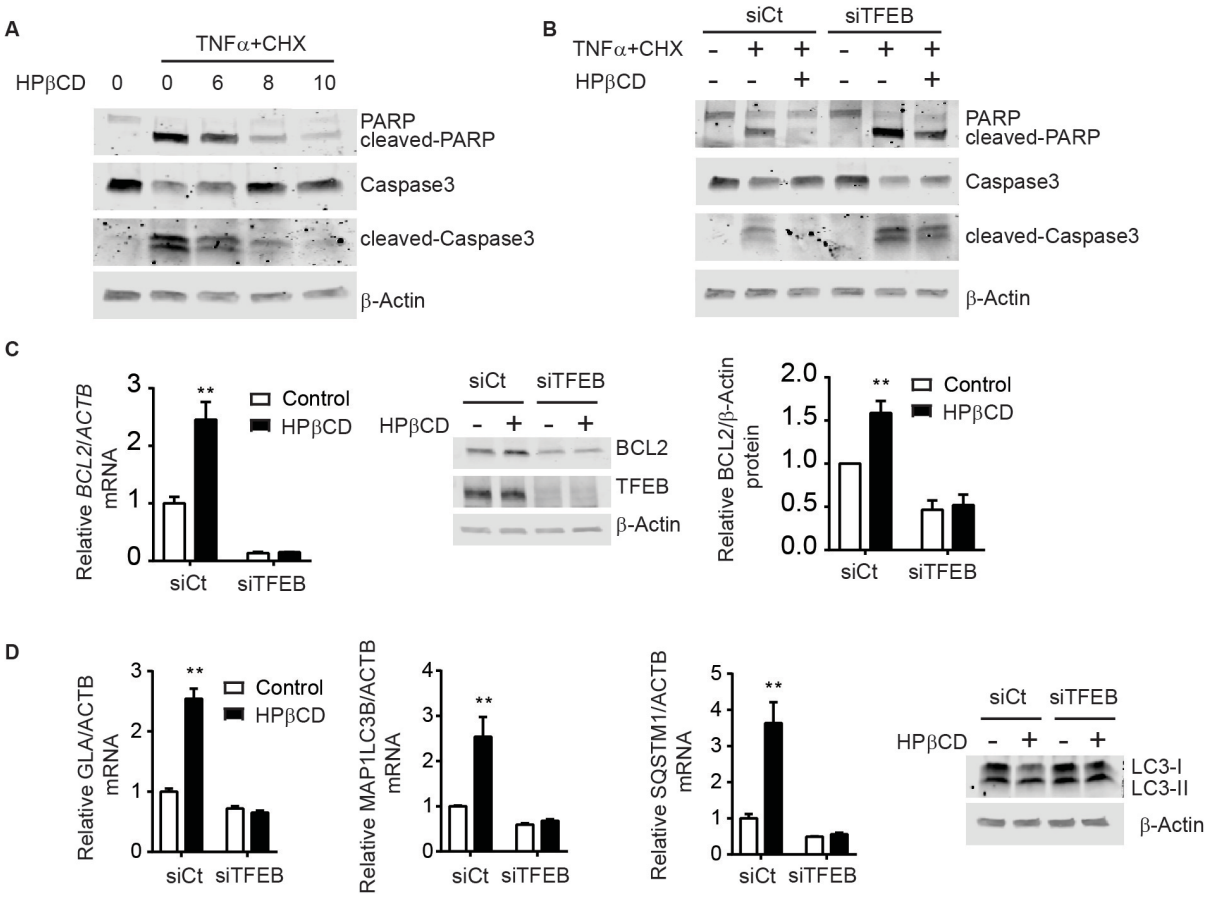

Figure 3.9 HPβCD inhibits HASMC dependent of TFEB.

(A) HASMCs were incubated with HPβCD at different dosage for 18 hours and then treated with TNFα+CHX for 6 hours. (B) HASMCs were transfected with siRNA negative control and siRNA against TFEB. After 48hours, cells were incubated with HPβCD (10mg/mL) for 18 hours and then treated with TNFα+CHX for 6 hours. (C-D) HASMCs were incubated with HPβCD (10mg/mL) for 24 hours and then mRNA and protein were determined by qPCR and Western blot respectively. * $p<0.05$ ** $p<0.01$ Data are presented as mean ± SEM of three independent experiments.

Discussion

In this study, we demonstrated that TFEB is down-regulated in the human aneurysm samples and is a key player in the formation and development of aortic aneurysm. The *in vitro* study has shown that TFEB is a crucial negative regulator of apoptosis in VSMCs. Although TFEB has emerged as an important regulator of lysosomal biogenesis and autophagy, the anti-apoptotic effect of TFEB in HASMCs cannot be attributed to this pathway, as inhibition of lysosome function and autophagy did not abolish it. Here, we found that BCL2 is a direct target of TFEB and BCL2 inhibitor abolishes the anti-apoptotic effect of TFEB overexpression. Using two different murine aneurysm models, we found that SMC specific *Tfeb* KO significantly increases VSMC apoptosis and aneurysm formation in the mouse. Based on this finding, we tested different TFEB activator in the murine aneurysm model and demonstrated that HPβCD attenuates aneurysm formation *in vivo* in a TFEB dependent manner. The mechanistic study also confirmed that HPβCD increases TFEB nuclear translocation in HASMC and increases the transcription of BCL2 as well as other TFEB target genes.

There have been numerous animal models reported for the study of the aneurysm.[190] Although these models all have their own drawbacks, they provide valuable tools to study the pathophysiology of the aneurysm and test the potential treatment before going to clinical trials. (1) Infusion of Ang II in $ApoE^{-/-}$ background is the most popular model to establish murine aneurysm, originally described in 2000[191]. The $ApoE^{-/-}$ background can be substituted with the injection of AAV encoding gain-of-function PCSK9 (D377Y)[177], which saves the time of the breeding work. This model recapitulates several important features of the human aneurysm, including atherosclerosis, thrombosis, lymphocyte infiltration, media dissection, and elastin network degradation[191]. However, the location of the aneurysm is limited to the suprarenal or the

ascending aorta, whereas human aortic aneurysms are usually in the infrarenal region. (2) Infusion of β-Aminopropionitrile (BAPN), a potent lysyl oxidase inhibitor, disrupts the crosslink of procollagens and tropoelastin and destroys the integrity of the aortic wall[192]. Co-treatment of Ang II and BAPN induces a high incidence of the aneurysm, with frequent rupture[193, 194]. The aneurysm can happen along the descending thoracic and abdominal aorta. The disruption of the elastic fiber occurs early, followed by neutrophil infiltration and VSMC apoptosis[190]. In this study, we employed both models to confirm the protective role of TFEB in the aortic aneurysm.

Apoptosis of the VSMCs was rare in the healthy aorta but become prominent during the development of aneurysm in human[195]. VSMC apoptosis has also been reported to be associated with aneurysm rupture in human[196]. The apoptosis can also be seen in various animal aneurysm models (CaPO4[168], BAPN[178, 197], ApoE/angiotensin II[198], elastase[199]). It is noteworthy that the administration of apoptosis inhibitor (pan-caspase inhibitor quinolone-Val-Asp-difluorophenoxymethylketone (Q-Vd-OPh)) starting before AngII infusion can significantly reduce aneurysm incidence and VSMC apoptosis in the ApoE/Ang II model[200]. All the evidence shows a clear causal relationship between VSMC apoptosis and aneurysm and targeting VSMC apoptosis remains a promising strategy to halt aneurysm development.

BCL2 family is the most important participator in the intrinsic apoptotic pathway. BCL2 was discovered in the human follicular lymphoma with the association with chromosomal translocation t(14;18)[201, 202]. Overexpression of BCL2 is capable of blocking cell death in various cell lines and primary cells[203, 204]. BCL2 family protein serves as a highly conservative mechanism to regulate cell death in the evolution[205] and acts at the convergence of the multiple upstreaming apoptotic pathways. BCL2 is localized to the nuclear envelope, the endoplasmic reticulum and the outer mitochondrial membrane in the cell[206]. BCL2 inhibits apoptosis by

binding to the pro-apoptotic BAK and BAX, preventing the mitochondrial outer membrane permeabilization (MOMP)[207]. BCL2 itself is also inhibited by several BH3-only proteins, such as BAD, BIK, BID. The relative abundance and interaction between pro- and anti-apoptotic BCL2 family protein determined the survival and death of the cell[208]. Although originally discovered as an oncogene, decades of study has shown that BCL2 is implicated in a variety of biological processes, such as kidney development, hair follicle cycling, neurodegenerative disorders, and angiogenesis[209]. Moreover, in the human aneurysmal lesion, BCL2 expression is decreased and related to the VSMC apoptosis[210, 211]. In this study, we found that TFEB deficiency reduces BCL2 expression and aggravates the VSMC apoptosis in murine aneurysm models, reinforcing the potential importance of BCL2 in the aneurysm. Notably, MITF, another member of the MITF family, has been shown to regulate BCL2 transcription in melanoma, indicating a similar pattern between different members in the MIT/TFE family[212, 213]. The anti-apoptotic effect of TFEB has been reported in other cell types, mainly in cancer cells[214, 215] , neurons[216] and cardiomyocytes[217, 218]. Here, we identified TFEB inhibits HASMC apoptosis in a novel BCL2-dependent but autophagy-independent pathway.

HPβCD is an FDA-proved agent to increase the solubility and delivery of hydrophobic drugs[219]. However, the potential pharmaceutical effect of HPβCD was not noticed until recently[220, 221]. HPβCD has shown a beneficial effect in Niemann-Pick Disease[222] and atherosclerosis regression[223]. HPβCD activates TFEB and promotes the clearance of ceroid lipopigment in fibroblasts[189]. In macrophages, HPβCD mediated TFEB activation suppresses M2 polarization in tumor microenvironment[49]. The biology of HPβCD could be due to its capacity to remove intracellular cholesterol and lipid. Lysosomal cholesterol depletion by methyl-cyclodextrin (another derivative of cyclodextrin) suppresses mTORC1 activation and

activates TFEB in HEK-293T cells[224]. Considering that HPβCD also can solubilize the cholesterol in the cell, it is reasonable to assume HPβCD activates TFEB also in a similar way as methyl-cyclodextrin[225]. Our finding further expanded the potential application of HPβCD in the clinics.

There are several limitations to this study. First, there may be difficulties in translating murine aneurysm study into clinical applications due to the species difference. Although HPβCD shows a promising effect on inhibiting the apoptosis of the human aortic smooth muscle cells, it still requires further investigation of the *in vivo* pharmaceutical effect of HPβCD. It is also of interest to elucidate the mechanism of how HPβCD activates TFEB in VSMCs. The regulation of TFEB mainly happens at the post-translational level, including phosphorylation and acetylation. Because of the sparse of the modification-specific TFEB antibody, it may require profiling by mass spectrometry. In addition, HPβCD exerts diverse biological activities in cells. We confirmed the TFEB-dependent effect of HPβCD in HASMCs by *in vitro* knockdown and *in vivo* KO experiment. However, we still cannot exclude HPβCD may also activate other pathways in HASMC, such as LXR[226].

In conclusion, we found that TFEB is decreased in the human aneurysm and TFEB negatively regulates VSMC apoptosis. SMC specific *Tfeb* KO promotes VSMC apoptosis and aneurysm formation in murine aneurysm models. Administration of TFEB activator HPβCD attenuates VSMC apoptosis and aneurysm formation in the mouse.

Chapter 4 Summary and Perspectives

Summary

Endothelial cell and vascular smooth muscle cells are two major residential cells in the vascular wall and maintain vascular homeostasis. In this dissertation work, we identified TFEB as an important regulator of the vascular wall biology in these 2 cell types. In the endothelial cells, TFEB is upregulated by protective laminar shear stress and mediates the anti-inflammatory effect of laminar shear stress in ECs, including TFEB is crucial to maintaining quiescent EC *in vivo*. By both gain-of-function and loss-of-function method, we demonstrated TFEB potently reduced endothelial cell inflammation *in vitro* and leukocyte adhesion *in vivo*. Using ApoE KO mice, we observed attenuated atherosclerotic development in EC specific *TFEB* transgenic mice. Notably, another group also reported the same anti-inflammatory effect of TFEB in EC in diabetic mice[42]. In VSMCs, we found that TFEB is downregulated in human aneurysm sample and TFEB could inhibit HASMC apoptosis via upregulation of BCL2. Based on this, we tested TFEB activator, HPβCD, and indeed found that HPβCD ameliorates the aneurysm formation in mice. These findings indicate activating TFEB could be an effective approach against cardiovascular diseases.

Perspective

TFEB as a master transcription factor regulating lysosomal biogenesis and autophagy. Autophagy is a house-keeping process for maintaining intracellular homeostasis via degradation

and recycling of proteins, lipids, and polysaccharides. Autophagy and lysosomal dysfunction have been implicated in several cardiovascular diseases, including atherosclerosis and cardiomyopathy. As a result, the restoration of autophagy and lysosomal function becomes a promising strategy to treat such diseases. TFEB regulates multiple steps in the process of autophagy and lysosomal function. In neuronal degenerative disease and lysosomal storage diseases, genetic or pharmacological activation of TFEB is able to attenuate the disease progression. Although less studied, TFEB overexpression or activation also shows a protective effect on atherosclerosis disease and cardiomyopathy. Activation of TFEB in vascular endothelial cell or macrophage by genetic or pharmacological strategies shows a promising beneficial effect on atherosclerosis and ischemia.

Although most studies focusing on the pro-autophagy role of TFEB in various cell types, there are also many studies indicating TFEB also regulates diverse pathways besides autophagy. TFEB promotes osteoblast differentiation by inhibiting activating transcription factor 4 (ATF4) and CCAAT/enhancer-binding protein homologous protein (CHOP)[227]. TFEB is a mediator of Ang II-induced skeletal muscle wasting by transcriptional control of muscle-enriched E3 ubiquitin ligase muscle RINGfinger-1 (MuRF1) expression[228]. In tumor-associated macrophages, the downregulation of TFEB promotes M2 polarization through suppressor of cytokine signaling 3 (SOCS3) signal transducer and activator of transcription 3 (STAT3) pathway[49]. In vascular endothelial cells, TFEB inhibits vascular inflammation and atherosclerosis via upregulation of anti-oxidative genes[229]. Considering that autophagy is a complicated multi-step indispensable process maintaining cell homeostasis, it is very hard to completely inhibit autophagy in the cells to study the autophagy-independent role of TFEB function, either genetically or

pharmacologically. However, the aforementioned studies shed lights on the comprehensive function of TFEB in different cell types under different conditions.

Current studies indicate that TFEB may play different roles in different tissue or cell types. It is crucial to understand what determines this cell-specific selection of target genes. The RNA-seq or ChIP-seq profiling could be used to explore the target genes of TFEB in different cell types. In mammalian cells, the initiation of transcription requires the recruitment of general RNA polymerase II machinery by multiple transcription activator and co-activators[230]. The cell type-specific expression pattern of the co-activators could determine the accessibility of TFEB to its target genes. The co-activators of TFEB are still not clear and the elucidation of these co-activators of TFEB could provide a means to manipulate TFEB activity in a specific cell type *in vivo*.

TFEB is mainly regulated by post-translational modifications and most TFEB activator targeted on the phosphorylation step. A myriad of pathways (mTOR, Akt, PKC, Ca^{2+}) has been manipulated by different chemicals to influence the phosphorylation and nuclear translocation of TFEB. However, it still remains challenging how these drugs would affect other downstream targets in the same pathway. This will require in-detailed studies to avoid unwanted off-target of these drugs to be used in the clinical trials. In addition, TFEB exerts diverse functions in different tissue, it is also of concern how to differentiate cell types in response to TFEB activator to avoid possible detrimental effect in certain cell type, such as potential tumorigenesis effect in renal cells[231].

Although this dissertation work has established that TFEB in endothelial cells and smooth muscle cells is an important player in the pathogenesis of vascular disease there are several unresolved areas for potential future research. First, it is still questionable if autophagy

plays some role in the protective effect of TFEB in vascular biology. Autophagy involves multiple protein complexes and organelles and there are several negative feedback pathways regulating autophagy process[232]. Systemic knockout of important autophagy genes leads to embryonic lethal or growth retardation, showing that autophagy is indispensable for development[233]. As a result, it is difficult to totally block autophagy *in vivo* to rule out the involvement of autophagy as a downstream pathway. Mice with tissue-specific KO of Atg7 have been reported and are available for the study of autophagy in various cell types *in vivo*[234]. This model could used to demonstrate if autophagy contributes to the phenotype caused by TFEB transgene or activation in mice. Second, there have always been challenges translating mouse studies to human diseases, particularly in cardiovascular disease research. For example, mice have heart rates that are ten times the normal resting heart of humans and are highly resistant to atherosclerosis due to high fat diet alone due to differences in cholesterol metabolism and LDL particle content. Therefore in some cases disease models in mice, for example that require loss of ApoE or genetic expression of PSCK9, cannot perfect mimic the human condition of acquired cardiovascular disease and there are evolutionary differences between the two specie[235]. No population genetic study shows a relationship between TFEB and any human diseases and this may be because of the importance of TFEB in embryonic development. Nevertheless, more work needs to be done to validate the mouse models and move the research from preclinical to clinical stages, perhaps using larger animals such as the rabbit or pig that can more readily develop atherosclerotic vascular disease by diet alone, and have cardiovascular physiology more similar to human.

Concluding remarks

In this dissertation, we provide evidence that TFEB is a crucial transcription factor to maintain vascular homeostasis. TFEB is responsive to the physiological (laminar shear stress) and pathophysiological (aneurysm) conditions in the vessel. TFEB overexpression or activation inhibits atherosclerosis and aortic aneurysm in mice. This study provides new insight into cardiovascular disease mechanisms and potential molecular target to treat human diseases.

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