Smooth muscle 22 alpha protein inhibits VSMC foam cell formation by supporting normal LXRα signaling, ameliorating atherosclerosis

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Vascular smooth muscle cells (VSMCs) are indispensable components in foam cell formation in atherosclerosis. However, the mechanism behind foam cell formation of VSMCs has not been addressed. We found a potential association between deletion of smooth muscle (SM) 22α and deregulated nuclear receptors liver X receptors (LXRs)/retinoid X receptor (RXR) signaling in mice. Here, we investigated the roles of SM22α in LXRα-modulated cholesterol homeostasis, and explore possible mechanisms underlying this process. We identified that the deletion of SM22α was a primary event driving VSMC cholesterol accumulation and the development of atherosclerosis in mice. Proteomic and lipidomic analysis validated that downregulation of SM22α was correlated with reduced expression of LXRα and ATP-binding cassette transporter (ABCA)1 and increased cholesteryl ester in phenotypically modulated VSMCs induced by platelets-derived growth factor (PDGF)-BB. Notably, LXRα was mainly distributed in the cytoplasm rather than the nucleus in the neointimal and Sm22α−/− VSMCs. Loss of SM22α inhibited the nuclear import of LXRα and reduced ABCA1-mediated cholesterol efflux via promoting depolymerization of actin stress fibers. Affinity purification and mass spectrometry (AP-MS) analysis, co-immunoprecipitation and GST pull-down assays, confocal microscopy, and stochastic optical reconstruction microscopy (STORM) revealed that globular-actin (G-actin), monomeric actin, interacted with and retained LXRα in the cytoplasm in PDGF-BB-treated and Sm22α−/− VSMCs. This interaction blocked LXRα binding to Importin α, a karyopherin that mediates the trafficking of macromolecules across the nuclear envelope, and the resulting reduction of LXRα transcriptional activity. Increasing SM22α expression restored nuclear localization of LXRα and removed cholesterol accumulation via inducing actin polymerization, ameliorating atherosclerosis. Our findings highlight that LXRα is a mechanosensitive nuclear receptor and that the nuclear import of LXRα maintained by the SM22α-actin axis is a potential target for blockade of VSMC foam cell formation and development of anti-atherosclerosis.

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INTRODUCTION

Vascular smooth muscle cells (VSMCs) are a major cell type present at intimal thickenings and all stages of an atherosclerotic plaque but with altered phenotypes [1]. The phenotypic switching of VSMCs, which is characterized by reduced myofilament density and contractile protein expression, is a key event during the development of atherosclerosis [2, 3]. Recently, the relative contribution of VSMCs to total foam cell formation has been determined in human atherosclerosis and in the model of mice [4, 5]. However, the mechanism underlying phenotypically switched VSMCs transforming into foam cells remains unclear.

Liver X receptors (LXRs) are members of the nuclear receptor superfamily of ligand-activated transcription factors and are primarily located in the nuclei with or without bound ligand and regulate the expression of genes involved in cholesterol metabolism and inflammation in a tissue-specific manner [6, 7]. The intranuclear concentration of LXRs is maintained by a balance between nuclear import, nuclear export, and nuclear retention. The regulation of this balance provides important regulatory mechanisms for transcription [8]. LXRα-driven expression of the cholesterol efflux transporter ATP-binding cassette transporter (ABCA)1 is essential for optimal reverse cholesterol transport in peripheral cells, which are associated with the pathogenesis of atherosclerosis [9]. VSMCs switched from contractile to synthetic phenotype metabolize lipids differently to contractile VSMCs, in part through decreased expression of ABCA1, resulting in an increased tendency to transform into foam cells [1, 6], but coordinated regulation of these processes has not been documented.
Smooth muscle (SM) 22α (also known as Transgelin), a differentiated VSMC marker [10], is involved in actin filament assembly and cytoskeletal rearrangements [11], which is required for maintaining the differentiated phenotype of VSMCs [12, 13]. The expression of SM22a has been demonstrated to be down-regulated in atherosclerosis and neointima, and further resulted in the depolymerization of the F-actin cytoskeleton, which represents phenotypic changes of VSMCs from the contractile to the synthetic [14, 15]. Loss of SM22a in hypercholesterolemic ApoE-deficient mice results in increased atherosclerotic lesion area and a higher proportion of proliferating SMC-derived plaque cells [16], implying that there is a potential causal relationship between SM22a depletion and atherosclerotic lesion. We and others have demonstrated that disruption of SM22a enhances the inflammatory response in VSMCs [17–19]. Conversely, overexpression of SM22a inhibits proliferation, inflammation, and oxidative stress of VSMC via blockade of different upstream pathways, and prevents neointima hyperplasia and aortic aneurysm formation [20–22]. Furthermore, these physiological and pathological effects of SM22a are mediated by the regulation of actin dynamics to some extent [16, 17]. Recently, the aortic transcriptome profiling suggests that SM22a knockout (Sm22a−/−) mice exhibited the characteristics of pro-atherosclerosis with deregulated nuclear receptors LXR/RXR (retinoid X receptor) and atherosclerosis signaling pathways [20]. Thus, we hypothesized that the protective effects of SM22a on vascular homeostasis may involve the regulation of LXR signaling.

In the current study, we first demonstrated that nuclear accumulation of LXRa is regulated by SM22a-modulated actin dynamics, and altered actin dynamics by SM22a depletion and the resulting inhibition of LXRa nuclear import accelerate the transformation of VSMCs into foam cells and development of atherosclerosis. Monomeric actin (G-actin) from F-actin depolymerization disrupts LXRa nuclear import and retains it in the cytoplasm. The normal LXRa signaling supported by SM22a is a potential target for blockade of VSMC foam cell formation and development of anti-atherosclerosis.

RESULTS
Depletion of SM22a contributes to the development of atherosclerosis in mice
We first took advantage of the published transcriptome analysis obtained on mouse aorta [23], to directly evaluate the sensitivity of Sm22a−/− mice to atherosclerosis. The levels of serum total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) gradually increased and were significantly higher in Sm22a−/− mice than that in wild-type (WT) mice when fed the Paigen diet for 24 weeks with no change of serum triglyceride (TG) levels (Supplementary Fig. 1a–c). The diet significantly increased aortic cholesterol and cholesteryl ester (CE) content in Sm22a−/− mice (Supplementary Fig. 1d). A positive oil red O staining was observed in aortic sinus sections of Sm22a−/− but not of WT mice when fed the Paigen diet, accompanied with diffuse vascular wall thickening (Fig. 1a, b). Moreover, Sm22a−/− Ldlr−/− mice on a Paigen diet displayed an aggravated atherosclerotic lesion compared to Ldlr−/− mice (Fig. 1a and Supplementary Fig. 1e), consistent with the previous finding [16].

Aortic stiffness is believed to be the earliest detectable manifestation of adverse structural and functional changes within the aortic wall [24]. Compared with WT mice, the aortic stiffness parameters, including elastic modulus, stiffness index, and reverse/forward flow ratio, were obviously increased in the outflow tract and aortic arch of Sm22a−/− mice fed Paigen diet, while aortic distensibility was decreased significantly compared with WT mice (Fig. 1c and Supplementary Fig. 1f). HE staining for the aortic sections also showed incomplete vascular structure and fragmentation of elastic fiber (Supplementary Fig. 1e) and a notable increase of fibrosis in the outflow tract and aortic arch of Sm22a−/− mice fed Paigen diet for 12 weeks (Supplementary Fig. 1g, h), which was earlier than the atherosclerotic lesion. The expression of collagen I α (Col1α) increased and elastin (Eln) decreased in the aortic tissues of Sm22a−/− mice (Supplementary Fig. 1i), suggesting that Sm22a−/− mice develop an aortic stiffness phenotype.

Bodipy staining showed that 50 ± 2% of foam cells costained strongly with smooth muscle marker α-actin (ACTA2) (Fig. 1d). Cells expressing both ACTA2 and CD68 that is a marker for macrophages as a percentage of total CD68+ cells were 12 ± 2% (n = 6) in the atherosclerotic lesions of Sm22a−/− mice (Fig. 1d), which was approximately one-fourth of the macrophages, consistent with that observed in the atherosclerotic lesions of ApoE−/− mice and human coronary artery [5]. The expression of LXRa and ABCA1 at mRNA and protein levels decreased in the aortic tissues from Sm22a−/− mice fed with Paigen diet or not, compared with WT mice (Supplementary Fig. 1j, k). Surprisingly, we observed numerous nuclear LXRa-staining negative VSMCs in the aortic sections from Sm22a−/− mice (Fig. 1e), suggesting that LXRa nuclear localization is disturbed. Overall, these data demonstrated that SM22a depletion induces atherosclerosis via a mechanism that involves the dysfunction of LXRa activity.

Expression and activity of LXRa are abnormal in Sm22a−/− VSMCs
We next mainly examined the effect of SM22a loss on the expression and distribution of LXRs in VSMCs as macrophages did not express SM22a [25]. The expression of LXRa was lower in Sm22a−/− VSMCs than that in WT cells under basic conditions, and no difference in LXRa expression was observed. T090- and cholesterol-induced LXRa expression was repressed in Sm22a−/− VSMCs compared to WT VSMCs (Fig. 2a and Supplementary Fig. 2a). To further establish the causal relevance of SM22a in the expression of LXRa, WT VSMCs were transfected with the specific siRNA of SM22a and Sm22a−/− VSMCs with Ad-GFP-Sm22a. We showed that knockdown of SM22a resulted in reduced expression of LXRa at mRNA and protein levels in WT VSMCs with or without cholesterol loading (Fig. 2b). Conversely, the downregulation of LXRa was significantly reversed by the rescue of Sm22a expression in Sm22a−/− VSMCs (Fig. 2c).

As VSMCs with negative nuclear LXRa-staining were found in the aortic sections of Sm22a−/− mice, we next determined whether the subcellular distribution of LXRa is changed in Sm22a−/− VSMCs. We showed that endogenous LXRa was mainly accumulated in the nucleus of WT VSMCs. However, nuclear LXRa was almost little in Sm22a−/− VSMCs, which exhibited diffuse cytoplasmic staining (Fig. 2d), and the ratio of cytoplasmic-/nuclear-LXRa protein markedly increased compared to WT controls (Supplementary Fig. 2b). The rescue of Sm22a expression restored endogenous LXRa nuclear localization in Sm22a−/− VSMCs (Fig. 2e), whereas the overexpression of LXRa-GFP was unable to do this, suggesting that LXRa nuclear accumulation is Sm22a-dependent and not influenced by LXRa level, and that defect of LXRa nuclear localization further reduces its expression, as LXRa is also a direct target gene of LXRa and enhances its own expression [26].

Activation of LXRa is also able to suppress collagen expression not only promotes transcription of lipid transport and, metabolism genes [27, 28], and VSMCs, as the main origin of extracellular matrix (ECM), are a significant regulator of ECM remodeling and arterial stiffness [29]. We showed that the mRNA level of ABCA1, ABCG1, and Eln was significantly downregulated (Fig. 2f), while the expression of Col1α was significantly elevated in Sm22a−/− VSMCs under basic and cholesterol loading conditions. By comparison, SM22a loss had little effect on the expression of collagen intakes genes LDL receptor (LDLR) and scavenger receptor class B type I (SR-BI). Cholesterol-induced dynamic upregulation of ABCA1 expression was observed from WT cells was missed in Sm22a−/− VSMCs (Fig. 2g), accompanied by increased cholesterol accumulation in a time-dependent manner (Fig. 2h). Reduced ABCA1
expression and increased cholesterol accumulation were verified in VSMCs with knockdown of SM22a (Supplementary Fig. 2c-e). Chromatin immunoprecipitation (ChIP) assay showed that the binding activity of LXRα to the promoter of abca1 and col1a genes was decreased in Sm22a−/− VSMCs compared to WT VSMCs (Fig. 2i, j), in accordance with decreased ABCA1 and increased Col1a expression. Rescue of SM22a decrease in Sm22a−/− VSMCs by transduction with Ad-GFP-SM22a increased in binding activity of LXRs to col1a promoter with a reduced Col1a expression (Supplementary Fig. 2f, g). Overall, these data indicate that LXRα transcriptional regulatory activity is repressed in Sm22a−/− VSMCs.

Not only does cholesterol deposits in foam cells at the atherosclerotic plaque, it also regulates cellular mechanics in atherosclerosis progression [30]. Next, the force-curve and Young's modulus of individual VSMCs were measured by atomic force microscopy (AFM). E-modulus of Sm22a−/− VSMCs was higher and significantly increased in response to cholesterol loading compared with WT cells (Fig. 2k), suggesting that cholesterol accumulation and substrate stiffness induce alternation of the biomechanics of VSMCs. Taken together, these results suggest that SM22a loss contributes to stiffness and foam cell formation of VSMCs.

Fig. 1 Impaired SM22a expression is associated with the development of atherosclerosis. a, b WT (n = 10) and Sm22a−/− (n = 10) mice with or without Ldlr−/− background (n = 10) fed Paigen diet for 8, 12 and 24 weeks respectively. Representative images of en face ORO-stained aortas (a), aortic sinus, aortic cross-sections (b) and quantification of lesion areas are shown. c M-mode and Doppler echocardiography images obtained from the aortic arch and outflow tract of WT (n = 15) and Sm22a−/− (n = 15) mice fed Paigen diet for 12 and 24 weeks. **p < 0.01; ***p < 0.001.

Fig. 2 Impaired SM22a expression is associated with cellular mechanics. a) M-mode and Doppler echocardiography images obtained from the aortic arch and outflow tract of WT (n = 6) and Sm22a−/− mice (n = 6) by CD68 (blue), ACTA2 (red) and Bodipy (green). Scale bar, 20 µm. Arrows indicated foam cells that were VSMCs-derived. b) Representative immunofluorescence of LXRs (red) and quantification of cells with nuclear LXRs in the aortic sections from WT (n = 3) and Sm22a−/− (n = 3) mice. Scale bar, 15 µm. Data and images are representative of at least three independent experiments. Data in (a) and (b) were analyzed by two-way and one-way ANOVA respectively. Data in (d) and (e) were analyzed by unpaired t test. *p < 0.05; **p < 0.01; ***p < 0.001.

Cell Death and Disease (2021) 12:982
Nuclear localization and signaling of LXRα are impaired in phenotypically switched VSMCs

SM22α loss is a prominent marker of phenotypic switching of VSMCs [10], and Sm22α−/− VSMCs have the characteristic of synthetic and pro-inflammatory phenotypes [17–19]. To determine whether the dysfunction of the LXRα-ABCA1 axis is common in phenotypically modulated VSMCs, we performed the proteomic analysis of the contractile and synthetic VSMCs and showed that the expression of ABCA1 was markedly reduced in the synthetic VSMCs (Fig. 3a, b and Supplementary Fig. 3a). We validated that
the expression of SM22α, ABCA1 and LXRα mRNAs decreased in a time-dependent manner in PDGF-BB-induced VSMCs (Fig. 3c). Notably, LXRα was gradually shifted from the nuclear to the cytoplasm in some VSMCs with extended PDGF-BB stimulation time (Fig. 3d). To determine whether nuclear localization of LXRα is impaired in modulated VSMCs in vivo, we examined VSMCs in the media and the neointima of mice. As expected, LXRα was mainly distributed in the nucleus of the medial VSMCs in the normal artery, whereas LXRα localization was shifted from the nucleus to the cytoplasm and merged with ACTA2 in the neointima VSMCs undergoing phenotypic modulation (Fig. 3e), indicating that LXRα nuclear localization was disturbed in these modulated VSMCs. To further confirm the effect of reduced LXRA-ABCA1 activity on lipid metabolism, the lipid profiles of VSMCs treated with PDGF-BB were
assessed by a lipidomic analysis. Comparison of the lipidome between the contractile and synthetic phenotypes showed that CE was significantly increased compared with other lipids (Fig. 3f and Supplementary Fig. 3b), which was a prominent feature of the lipid profiles of synthetic VSMCs (Fig. 3g). Moreover, increased cholesterol deposition was also validated in PDGF-BB-induced VSMCs by ORO staining (Fig. 3h). Overall, these data further suggest that nuclear localization and signaling of LXRs are impaired during phenotypic switching of VSMCs, associated with the depletion of SM22α.

Targeting SM22α supports normal LXRa signaling and ameliorates atherosclerosis

To further identify a potential causative link between SM22α expression and LXRa signaling, we selected an AAV carrying SM22α to perform a gain-of-function study in Sm22α−/− mice fed Paigen diet. As expected, the administration of AAV-SM22α restored SM22α expression in the aortic wall of Sm22α−/− mice accompanied with enhanced aortic GFP signal, indicating a high efficiency of viral transfection (Supplementary Fig. 3c-e). Compared with AAV-GFP mice, the administration of AAV-SM22α significantly reduced Paigen diet-induced aortic stiffness and atherosclerotic lesion (Fig. 3i-j and Supplementary Fig. 3d-e), indicating that AAV-SM22α intervention is effective. qRT-PCR showed that Col1α expression was decreased and the expression of Eln, LXRa and ABCA1 was elevated in the aortic wall of Sm22α−/− mice with AAV-SM22α (Supplementary Fig. 3f), accompanied with reduced expression of MCP-1, MPP2, MPP9, VCAM-1 and ICAM-1 that are downstream of NF-κB, as LXRa has been reported to inhibit NF-κB activity [31]. Importantly, LXRa was mainly accumulated in the nucleus of VSMCs (Fig. 3k), and VSMC marker positive foam cells were reduced in the aortic arch of Sm22α−/− mice with AAV-SM22α (Fig. 3l). Collectively, these findings suggest that SM22α ameliorates atherosclerosis via supporting nuclear localization of LXRa.

Nuclear import of LXRa is regulated by actin dynamics

To elucidate the mechanism underlying cytoplasmic retention of LXRa, nuclear import of LXRa-GFP was measured by fluorescence loss in photobleaching (FLIP) and fluorescence recovery after photobleaching (FRAP) experiments. In WT cells, nuclear import of LXRa-GFP was extremely rapid, being effectively complete within 2 min (Fig. 4a and Supplementary Movie 1) and dramatically delayed and reduced in Sm22α−/− cells (Supplementary Movie 2). LXRa phosphorylation and the formation of a heterodimer with retinoid X receptor (RXR) have been demonstrated to be required for LXRa nuclear accumulation [32, 33]. We showed that SM22α loss did not affect the phosphorylation of LXRa (Supplementary Fig. 4a), and also had no effect on the interaction between LXRa and RXRa (Supplementary Fig. 4b).

To investigate the possible sequestering proteins that retard LXRa nuclear import, we performed affinity purification using an anti-LXRa antibody, and the precipitates were subjected to mass spectrometry analysis. A total of 48 proteins potentially interacting with LXRa was identified (Supplementary Table). Next, we used GO enrichment analysis to cluster and characterize these proteins according to their biological processes (Supplementary Fig. 4c). Ultimately, the actin associated with muscle cell differentiation was selected as a candidate for its reported function in guiding transcriptional factors nuclear transport [34, 35]. As disruption of SM22α promotes actin cytoskeleton remodeling in VSMCs [17, 22], we subsequently compared actin organization between Sm22α−/− and WT cells. Sm22α−/− VSMCs exhibited less fraction of bundled stress fibers (Fig. 4b), and a higher total G-actin and parallel reduced ratio of F-actin to G-actin compared to WT cells (Supplementary Fig. 4d). Moreover, LXRa was mainly distributed in the perinuclear area of the cytoplasm with less F-actin in Sm22α−/− VSMCs (Fig. 4c). To validate whether altered actin organization is associated with impaired nuclear translocation of LXRa, VSMCs were treated with jaspilkinolide (JPK) which stabilizes F-actin and cytochalasin-B (CytoB) that inhibits actin polymerization. We showed that LXRa almost entirely remained in the cytoplasm of VSMCs treated by CytoB (Fig. 4d), with a decreased ratio of F-actin to G-actin (Supplementary Fig. 4d), and JPK did not affect the nuclear accumulation of LXRa. CytoB washout restored actin dynamics and distribution of LXRa in the nucleus (Fig. 4d and Supplementary Fig. 4d). Western blot for LXRa expression in the nuclear and cytoplasm fractions showed that LXRa protein was gradually shifted from the nucleus to the cytoplasm during CytoB treatment for different times, with increased cytoplasm/nucleus ratio of LXRa (Fig. 4e). Together, these data for the first time demonstrated that LXRa is a mechanosensitive nuclear receptor and that nuclear translocation of LXRa is regulated by actin dynamics.

G-actin directly interacts with and retains LXRa in the cytoplasm

To test whether LXRa directly interacts with actin in vivo, we performed computational docking analysis for G-actin and LXRa via the ZDOCK server and discovered the highest-scored predicted model of interaction between them (Supplementary Fig. 4e). As LXRa did not colocalize with the F-actin cytoskeleton (Fig. 4c), we used Dnasel to label G-actin and observed that G-actin colocalized with LXRa in the cytoplasm of Sm22α−/− and CytoB-treated VSMCs (Fig. 5a). Next, F-actin and G-actin fractions isolated from WT and Sm22α−/− VSMCs were subjected to co-immunoprecipitation (co-IP) with specific anti-ACTA2 and anti-LXRa antibodies. A specific LXRa band was present in the complex immunoprecipitated by the anti-ACTA2 antibody in the G-actin fraction but not in the F-actin fraction of Sm22α−/− but not WT VSMCs (Fig. 5b). Meanwhile, ACTA2 did not co-immunoprecipitate with LXRB in both F-actin and G-actin fractions of Sm22α−/− VSMCs (Fig. 5c). Importantly, an atherosclerotic lesion assay using Dnasel and anti-LXRa antibodies revealed a colocalization between endogenous LXRa and G-actin in the aortic wall of Sm22α−/− mice (Fig. 5d). To further validate that increased G-actin level contributes to the retention of LXRa in the cytoplasm, we overexpressed HA-ACTA2 in WT VSMCs and showed that Dnasel-stained G-actin markedly increased (Supplementary Fig. 5a). Importantly, endogenous LXRa redistributed from the nucleus to the cytoplasm and colocalized with ACTA2 in the VSMCs (Fig. 5e), and exogenous LXRa-GFP was so in co-expressed cells. Similar results were observed in HEK-293A cells co-expressing LXRa-GFP and HA-ACTA2, different from the cells expressing LXRa-GFP alone that had nuclear LXRa-GFP fluorescence (Fig. 5f). Thus, G-actin is a novel inhibitor of LXRa nuclear accumulation.

To gain direct insight into G-actin-LXRa interaction in cells, we exploited stochastic optical reconstruction microscopy (STORM) [36], a super-resolution imaging method, to examine the spatial distributions of the two proteins. In WT cells, an obvious much broader peak was observed for the nearest-neighbor distribution of LXRa and G-actin (Fig. 5g). Upon treatment with CytoB, STORM displayed a significant reduction in the nearest-neighbor distance between LXRa and G-actin (Fig. 5h). A quantitatively similar increase in colocalization between LXRa and G-actin was observed using Sm22α−/− VSMCs (Fig. 5i). Such colocalization was abolished by the rescue of Sm22α expression (Fig. 5j). Together, these findings suggest that LXRa is recruited by G-actin.

G-actin binding disturbs interaction between LXRa and Importin α

Binding to Importin α that is to serve as an adaptor is the first step in the nuclear transport of nuclear receptors by that link them to Importin β to form a ternary complex in the cytoplasm [37]. We observed that neither CytoB treatment nor loss of SM22α changed Importin α expression (Fig. 5k). Notably, the expression of Importin α
α in the LXRα-immunoprecipitated complex decreased evidently or even disappeared in Sm22α−/− VSMCs compared to WT cells, which was returned the level to WT cells via the rescue of SM22α expression (Fig. 5k). Similarly, the LXRα-importin α complex was decreased in CytoB and PDGF-BB-treated WT VSMCs, accompanied with reduced nuclear LXRα expression (Supplementary Fig. 5b), indicating that Importin α-mediated LXRα nuclear import was inhibited by altered actin dynamics, associated with VSMC

**D.-D. Zhang et al.**

Cell Death and Disease (2021) 12:982
Fig. 4   Nuclear import of LXRα is regulated by actin dynamics. a Fluorescence recovery after photobleaching (FRAP) studies with LXRα-GFP to measure nuclear import. Cells were pretreated with LMB. Decreased accumulation of nuclear fluorescence indicates a lower rate of nuclear import of LXRα-GFP in Sm22α−/− VSMCs relative to WT controls (n = 25). b Representative images of F-actin (phalloidin, red) and G-actin (DnaseI, green) in WT and Sm22α−/− VSMCs. Scale bar, 10 μm. c, d Representative images for F-actin (phalloidin, red) and LXRα (green) in WT and Sm22α−/− VSMCs with cholesterol loading or not (c) and in WT VSMCs treated with JPK, CytoB and after CytoB washout (d). Scale bars, 10 μm. e Western blot analysis of cytoplasmic and nuclear LXRα binding to Importin α or ACTA2 in WT and Sm22α−/− VSMCs relative to WT controls (n = 3). f Co-immunoprecipitation of ACTA2 and LXRα (b) and LXRα (c) respectively in F- and G-actin fractions of WT and Sm22α−/− VSMCs transfected with HA-ACTA2 (red, stained by anti-HA antibody) or not. Scale bar, 20 μm. g Representative immuno-fluorescence staining for endogenous LXRα (green) or IgG in the atherosclerotic lesion in the aortic wall of Sm22α−/− mice. Scale bar, 10 μm. h, i Double immuno-fluorescence staining for LXRα-GFP (green) and HA-ACTA2 (red, stained by anti-HA antibody) or not. Scale bar, 15 μm. j Western blot analysis of cytoplasmic and nuclear LXRα/β, Improtin α−/−/β−/−, and Sm22α−/− VSMCs with cholesterol loading or not (c) and in WT VSMCs treated with JPK, CytoB and after CytoB washout (d). Scale bars, 10 μm. k, l Double immuno-fluorescence staining for G-actin (DnaseI, red) and LXRα (green) or IgG in the atherosclerotic lesion in the aortic wall of Sm22α−/− mice. Scale bar, 10 μm. m Western blot analysis of cytoplasmic and nuclear LXRα/β, Improtin α−/−/β−/−, and Sm22α−/− VSMCs with cholesterol loading or not (c) and in WT VSMCs treated with JPK, CytoB and after CytoB washout (d). Scale bars, 10 μm. n Co-immunoprecipitation of LXRα and Importin α, Improtin β or ACTA2 in WT and Sm22α−/− VSMCs with or without JPK, CytoB, PDGF-BB and Ad-GFP-SM22α treatment (n = 3). o Double immuno-fluorescence staining for Importin α (red) and LXRα (green) in WT and Sm22α−/− VSMCs as well as CytoB-treated WT VSMCs. Scale bar, 15 μm. p Co-immunoprecipitation of LXRα and Importin α, Improtin β or ACTA2 in WT VSMCs transfected with HA-ACTA2 of different concentrations (n = 3). Data and images represent at least three independent experiments.

Fig. 5   G-actin interacts with and retains LXRα in the cytoplasm, blocking LXRα binding to Importin α. a Double immuno-fluorescence staining for G-actin (DnaseI, red) and LXRα (green) in WT VSMCs accompanied with the treatment of JPK or CytoB and also in Sm22α−/− VSMCs. Scale bar, 10 μm. b, c Co-immunoprecipitation of ACTA2 and LXRα (b) and LXRα (c) respectively in F- and G-actin fractions of WT and Sm22α−/− VSMCs (n = 3). d Double immuno-fluorescence staining of G-actin (DnaseI, red) and LXRα (green) or IgG in the atherosclerotic lesion in the aortic wall of Sm22α−/− mice. Scale bar, 20 μm. e Representative immuno-fluorescence staining for endogenous LXRα (green) and LXRα-GFP (green) in WT VSMCs transfected with HA-ACTA2 (red, stained by anti-HA antibody) or not. Scale bar, 15 μm. f Representative immuno-fluorescence staining for LXRα-GFP (green) and HA-ACTA2 (red, stained by anti-HA antibody) in HEK-293A cells. Scale bar, 10 μm. g–j Two-color STORM images and quantification of the colocalization degree between LXRα and G-actin as well as Importin α in WT VSMCs with (h) or without (g) CytoB treatment and Sm22α−/− VSMCs with (j) or without (i) Ad-GFP-SM22α infection (n > 10). k Co-immunoprecipitation of LXRα and Importin α, Importin β or ACTA2 in WT and Sm22α−/− VSMCs with or without JPK, CytoB, PDGF-BB and Ad-GFP-SM22α treatment (n = 3). l Double immuno-fluorescence staining for Importin α (red) and LXRα (green) in WT and Sm22α−/− VSMCs as well as CytoB-treated WT VSMCs. Scale bar, 15 μm. m Co-immunoprecipitation of LXRα and Importin α, Importin β or ACTA2 in WT VSMCs transfected with HA-ACTA2 of different concentrations (n = 3). Data and images represent at least three independent experiments.
phenotypes. These results were verified by immunofluorescence staining and confocal analysis (Fig. 5I). Next, we used STORM to examine the spatial relationship of LXRα to Importin α. In WT VSMCs, LXRA colocalized with Importin α, which was reflected by a nearest-neighbor distribution with a sharp peak near the 10–50 nm theoretical resolution limit of STORM (Fig. 5g), whereas disruption of F-actin by CytoB treatment or SM22α knockout completely eliminated this natural colocalizations (Fig. 5h, i), which displayed increased dramatically the nearest-neighbor distribution. The degree of colocalization between LXRA and Importin α was similar to WT cells upon the rescue of SM22α expression (Fig. 5j). This finding was supported by co-immunoprecipitation experiments (Fig. 5m). Taken together, these results suggest that G-actin acts as a molecular shield against LXRA binding to Importin α.

The C-terminal domain mediates the interaction between G-actin and LXRA

To characterize which part of ACTA2 is responsible for LXRA interaction, we then reconstructed the two structural domains of ACTA2 with HA-tagged truncated N-terminus (HA-ACTA2-NT, aa. 1–140) and C-terminal domains (HA-ACTA2-CTD, aa. 141–377) (Supplementary Fig. 6a), and used different truncation derivatives of ACTA2 to gain insight into the location of the interaction. The cells expressing HA-ACTA2-CTD displayed increased LXRA-GFP fluorescence intensity in the cytoplasm and reduced nuclear localization of LXRA-GFP (Fig. 6a) like HA-ACTA2-overexpressed cells (Fig. 5f). Furthermore, the ACTA2-CTD, but not ACTA2-NT, colocalized with LXRA-GFP in the cytoplasm. Similarly, in HEK293A cells co-expressing LXRA-GFP and ACTA2 truncation derivatives, the ACTA2-CTD colocalized with LXRA-GFP in the cytoplasm and abolished nuclear LXRA-GFP, whereas the ACTA2-NT did not influence the nuclear accumulation of LXRA-GFP (Fig. 6b), suggesting that ACTA2-CTD predominantly contributed to this interaction and retarded nuclear import of LXRA.

Because there were no RPEL motifs that bind to actin in LXRA sequence [38], in turn, we constructed the two truncated mutants of LXRA-N-terminus (NT, aa. 1–170) that contained the DNA binding domain and three nuclear localization sequences (NLSs) (NLS1, 2 and 4) and LXRA-C-terminal domains (CTD, aa. 171–445) that included the hinge region, one NLS (NLS3) and the putative ligand-biding domain (Supplementary Fig. 6b) [38, 39], and transfected them into Sm22α−/− VSMCs. We showed that only LXRA-NT was accumulated in the nucleus, and LXRA-CTD, like endogenous LXRA, was also trapped in the cytoplasm of Sm22α−/− cells (Fig. 6c). Similarly, nuclear localization of only LXRA-NT was observed in HEK293A cells co-expressing HA-ACTA2-CTD, whereas LXRA-CTD colocalized with ACTA2-CTD in the cytoplasm (Fig. 6d). Next, peptide pull-down experiments with recombinant purified GST-LXRA, GST-LXRA-NT, and GST-LXRA-CTD revealed that both GST-LXRA and GST-LXRA-CTD bound directly to ACTA2 and ACTA2-CTD rather than ACTA2-NT (Fig. 6e). Thus, LXRA-CTD is the region for ACTA2 recognizing and binding to LXRA. Together, these data suggest that the C-terminal domains mediate the interaction between G-actin and LXRA.

**DISCUSSION**

In the present study, we showed that the depletion of SM22α dysregulated LXRA signaling and promoted foam cell formation of VSMCs and the development of atherosclerosis. G-actin interacted with LXRA and inhibited its nuclear import, as the complex blocked LXRA binding to Importin α. SM22α regulated the nuclear localization of LXRA through a mechanism in which F-actin polymerization by SM22α led to dissociation of this complex (Fig. 6f). Using Sm22α−/− and Sm22α−/− Ldlr−/− mice, we provide evidence for a causative role of SM22α loss in LXRA signaling and VSMCs foam cell formation. G-actin was identified as a negative regulator of the LXRa nuclear import and activity.

The disruption of LXRa is believed to be an important factor in the pathological development of atherosclerosis via leading to foam cell formation in macrophages of the arterial wall [9, 40, 41]. Though the expression of LXRa is lower in human VSMCs, limited studies have demonstrated that LXRa can influence proliferation, contractility, apoptosis, and calcification in VSMCs [42]. Moreover, ABCA1 expression is reduced in neointimal VSMCs compared with those isolated from the medial layer [43], more so in advanced relative to early atherosclerosis [44]. In the current study, similar atherosclerotic phenotypes to those of LXRa-deficient mice were observed in Sm22α−/− mice in the context of hypercholesterolemia. The diffuse thickenings of the vascular walls and aortic stiffness existed in Sm22α−/− mice on a Paigen diet for 12 weeks, which are widely considered the most likely precursor of atherosclerotic plaques [1, 45]. Rescue of SM22α expression could alleviate cholesterol overload and displayed anti-atherogenic effects that presented as reduced aortic stiffness and lesion area. Interestingly, we demonstrated that one different aspect from the study on Lxrα−/− mice is that the increased atherosclerosis in Sm22α−/− mice is associated with an inability of VSMCs rather than macrophages to efficiently efflux cholesterol through the LXR pathway. More importantly, the observation that nuclear import of LXRa was impaired in VSMCs of Sm22α−/− mice was removed by the rescue of SM22α expression in vitro and in vivo. Our findings suggest a particularly important role for SM22α in LXRa-mediated cholesterol homeostasis and contractile phenotype in VSMCs especially in the context of hypercholesterolemia and provide evidence that SM22α contributes to the anti-atherogenic effects of LXRa on VSMCs.

De-differentiation, modulation or phenotype switching of VSMCs is characterized by reduced myofilament density and lower expression of contractile proteins [1]. It is known that modulated VSMCs predominate in the thickened arterial intima at atherosclerosis-prone sites prior to the onset of plaque formation and VSMC foam cell formation is resulted from modulated VSMCs engulfing oxidized low-density lipoprotein [46]. In the present study, the proteomic and lipidomic analysis showed that SM22α loss correlated with reduced LXRa-ABCA1 expression and increased cholesteryl ester in phenotypically modulated VSMCs. We validated that LXRa was redistributed from the nuclear to the cytoplasm in VSMCs upon PDGF-BB stimulation and in the neointima VSMCs and that LXRa colocalized with G-actin in the cytoplasm, suggesting that LXRa nuclear localization is regulated by actin dynamics and is impaired as a result of VSMC phenotypic switching. Our findings indicated that SM22α loss-mediated aberrant actin-LXRa signaling pathway guides modulated VSMCs to ultimately transform into foam cells. Our results support the idea that lipid metabolism programming is a critical event in phenotypic switching of VSMCs and that SM22α activates the LXRa-ABCA1 axis to maintain lipid homeostasis through the modulation of cytoskeletal actin polymerization.

We acknowledge several limitations of this study. First, SM22α is not only expressed in SMCs but also expressed in other lineages, such as cardiomyocytes during development and myeloid cells [47]. Therefore, using inducible SMC-specific SM22α knockout mice are warranted to accurately define a more definitive causal relationship between SM22α expression in VSMCs and their contribution to atherosclerotic lesion formation. Second, polymerization and depolymerization in live cells are regulated by actin-binding proteins. It needs further to explore whether and how other actin-binding proteins are involved in the regulation of LXRa signaling and cholesterol metabolism. Finally, given the involvement of the actin cytoskeleton remodeling in phenotypic switching of VSMC, it will be important to determine how these and similar pathways of aberrant actin-to-LXRa crosstalk can be intervened by the development of therapeutic agents.
Fig. 6 The C-terminal domain mediates the interaction between G-actin and LXRα. a Representative immunofluorescence staining for LXRα-GFP (green) in WT VSMCs transfected with HA-ACTA2-CTD (red) or HA-ACTA2-NT (red). Scale bar, 15 μm. b Representative immunofluorescence staining for LXRα-GFP (green) and HA-ACTA2-CTD (red) or HA-ACTA2-NT (red) in HEK-293A cells. Scale bar, 15 μm. c LXRα-CTD-GFP (green) or LXRα-NT-GFP (green) was transfected into Sm22α−/− VSMCs. Scale bar, 10 μm. d LXRα (-CTD, -NT)-GFP (green) and HA-ACTA2-CTD (red) were co-expressed in HEK-293A cells. Scale bar, 10 μm. e Interaction of HA-ACTA2 (-FL, -CTD, -NT) and GST-LXRα (-FL, -CTD, -NT) proteins analyzed by in vitro pull-down assay (n = 3). f Schematic representation of a working model in which SM22α inhibits VSMC-derived foam cell formation by blocking actin-LXRα signaling ameliorating atherosclerosis. Data and images represent at least three independent experiments.
DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

2. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

3. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

4. Allahverdian S, Chehroudi AC, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

5. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

6. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

7. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

8. Allahverdian S, Chehroudi AC, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

9. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

10. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

11. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

12. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

13. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

14. Shahman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

15. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

16. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

17. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

18. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

19. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

20. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

21. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

22. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

23. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

24. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

25. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

26. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

27. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

28. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

29. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

30. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

31. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

32. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

33. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

34. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

35. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

36. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

37. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

38. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

39. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

40. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

41. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

42. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.

43. Basatemur GL, Jrgensen HF, Clarke MCH, Bennett MR, Mallat Z. Vascular smooth muscle cells in atherosclerosis. Nat Rev Cardiol. 2019;16:727–44.

44. Glass CK, Witztum J. Atherosclerosis. The road ahead. Cell. 2001;104:503–16.

45. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature. 2011;473:317–25.

46. Shankman LS, Gomez D, Cherepanova OA, Salmon M, Alencar GF, Haskins RM, et al. KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque progression. Nat Med. 2015;21:628–37.
AUTHOR CONTRIBUTIONS
MH initiated the project and designed the paper. D-DZ, YS, and PK performed most of the experiments and/or analyzed data. Y-KG, XX, X-WW, and Y-LL assisted in animal experiments. LW, Y-QD, and FZ assisted in in vitro experiments and histological analyses. PK and D-DZ generated the illustrations. MH wrote the manuscript and edited the manuscript. All authors read and approved the final manuscript.

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COMPETING INTERESTS
The authors declare no competing interests.

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