KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis

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Previous studies investigating the role of smooth muscle cells (SMCs) and macrophages in the pathogenesis of atherosclerosis have provided controversial results owing to the use of unreliable methods for clearly identifying each of these cell types. Here, using Myh11-CreER12 ROSA floxed STOP eYFP Apoe−/− mice to perform SMC lineage tracing, we find that traditional methods for detecting SMCs based on immunostaining for SMC markers fail to detect >80% of SMC-derived cells within advanced atherosclerotic lesions. These unidentified SMC-derived cells exhibit phenotypes of other cell lineages, including macrophages and mesenchymal stem cells (MSCs). SMC-specific conditional knockout of Krüppel-like factor 4 (KLF4) resulted in reduced numbers of SMC-derived MSC- and macrophage-like cells, a marked reduction in lesion size, and increases in multiple indices of plaque stability, including an increase in fibrous cap thickness as compared to wild-type controls. On the basis of in vivo KLF4 chromatin immunoprecipitation–sequencing (ChIP-seq) analyses and studies of cholesterol-treated cultured SMCs, we identified >800 KLF4 target genes, including many that regulate pro-inflammatory responses of SMCs. Our findings indicate that the contribution of SMCs to atherosclerotic plaques has been greatly underestimated, and that KLF4-dependent transitions in SMC phenotype are critical in lesion pathogenesis.

Atherosclerosis is a disease of chronic inflammation and is the leading cause of morbidity and mortality worldwide. There is a general consensus that the majority of coronary syndromes result from the rupture of unstable plaques and associated thrombotic events1–3. Plaque instability has been associated with disruption of the fibrous cap, an atheroprotective layer of smooth muscle composed primarily of phenotypically modulated SMCs 9,10. Previous studies showing that SMCs and macrophages are often misidentified within human advanced coronary lesions come from studies of cross-gender bone marrow transplant subjects. These studies have shown that >10% of ACTA2+ cells within lesions are of hematopoietic stem cell (HSC) and not SMC origin14. Consistent with these human data, a substantial fraction of the cells that express SMC markers (including ACTA2 but not MYH11) within lesions of Apoe−/− mice are of HSC and not SMC origin15.

Conversely, there is extensive evidence suggesting that many SMC-derived cells within advanced lesions of Apoe−/− mice lack detectable expression of conventional SMC markers such as ACTA2 (ref. 16), and/or activate expression of macrophage markers17. Notably, in vivo studies from our laboratory showed that large numbers of ACTA2−, myosin, heavy polypeptide 11, smooth muscle (MYH11)−, and transgelin (TAGLN)− cells within advanced lesions of Apoe−/− Western diet–fed mice retain expression of a mutant Tagln-lacZ transgene that is resistant to downregulation compared to the unmutated transgene16. Unfortunately, these studies are not definitive because we could not rule out the possibility that non-SMCs present within lesions may activate the mutant transgene.

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Thus, despite decades of atherosclerosis research, we still do not know which cells within lesions are SMC-derived and to what extent they contribute to lesion pathogenesis. A recent SMC lineage tracing study using a Tagln-ERT²Cre-lacZ Apc⁻/⁻ mouse model provided evidence that SMC-derived cells within advanced lesions activate some macrophage markers, including LGALS3 and CD68 (ref. 17). Unfortunately, as highlighted in an editorial on this paper, the labeling efficiency of SMCs in this study was only 11%, precluding a determination of the fraction of macrophage-like cells within lesions that are derived from SMCs or, most importantly, how these cells might contribute to lesion pathogenesis. Another recent study showed that 50% of foam cells within advanced human coronary artery lesions express the SMC marker ACTA2, highlighting the magnitude of the ‘SMC/macrophage misidentification problem’ with respect to our understanding of human disease. However, the majority of these ACTA2⁺ foam cells also expressed the macrophage marker CD68 and represented 40% of all CD68⁺ lesional cells. Given clear evidence that macrophages can activate SMC markers and, vice versa, that SMCs can activate macrophage markers, it is unclear whether these CD68⁺ lesional cells are derived from SMCs, macrophages, or another cell type.

In view of the major ambiguities in identifying which cells within atherosclerotic lesions are SMC-derived versus macrophage-derived, the most crucial questions are: (i) how are the phenotypic transitions of SMCs, macrophages, and other cell types regulated within lesions; (ii) what is the function of these phenotypically modulated cells; and (iii) how do these phenotypic transitions affect overall disease pathogenesis? To begin to address these questions, we generated atherosclerosis-prone Apc⁻/⁻ mice with which we could lineage-trace SMCs and study the effects of SMC-specific conditional knockout of the stem cell pluripotency gene, KLF4. We chose to study the role of KLF4 as we and others have previously shown that this transcription factor plays a key role in regulating phenotypic transitions of SMCs in vivo during development and after carotid ligation injury, as well as in vitro in cultured SMCs treated with platelet-derived growth factor (PDGF)-BB, PDGF-DD or oxidized phospholipids.

RESULTS

Most atherosclerotic plaque SMCs are not identified by ACTA2

SMCs are distinguished from other cell types by expression of a unique repertoire of genes including Acta2, Tagln, and Myh11. These genes are coordinately downregulated, at least in vitro, during SMC phenotypic switching such that they may be undetectable using traditional immunohistochemical staining methods. Therefore, to rigorously analyze the overall contributions of SMCs to lesion pathogenesis, we used a previously described Myh11-CreERT² ROSA floxed STOP eYFP Apc⁻/⁻ (SMC YFP⁺Apc⁻/⁻) mouse model in which >95% of medial SMCs within large arteries were labeled with YFP (Supplementary Fig. 1a). Because Cre excision is permanent, this SMC lineage-tracing model provides permanent YFP lineage tagging of virtually all mature (MYH11⁺) arterial SMCs that exist at the time of tamoxifen injection, allowing study of the further differentiation of these cells or of their progeny, irrespective of continued expression of Acta2, Myh11, or other SMC marker genes. To ensure the fidelity and SMC specificity of this lineage tracing model, we completed a number of further validation studies beyond those shown in our previous studies. We demonstrated (i) SMC-specific YFP labeling within all tissue specimens examined, with no detectable expression of the YFP indicator gene in the absence of tamoxifen (Supplementary Figs. 1a,b and 2a); (ii) no detectable YFP⁺ cells by flow cytometry within blood or bone marrow preparations (Supplementary Fig. 2a); (iii) no evidence of YFP⁺ cells within lesions of Western diet–fed mice not given tamoxifen (Supplementary Fig. 2b); (iv) no detectable YFP⁺ cells in the blood of the mice when fed a high-fat diet for 18 weeks (Supplementary Fig. 2c); and (v) YFP⁺ labeling of approximately 60% of freshly enzymatically dissociated cells from the aorta (the ascending and descending thoracic aorta plus the abdominal aorta to the iliac bifurcation, including adventitial and intimal cells) on the basis of flow cytometric analysis (Supplementary Fig. 2d).

We harvested brachiocephalic arteries (BCAs) from SMC YFP⁺/⁻ Apc⁻/⁻ mice that had been injected with tamoxifen between 6–8 weeks of age, after an additional 18 weeks of Western diet. BCAs were immunostained for YFP, ACTA2, and LGALS3. Confocal microscopy z-stacks of the collected tissues were acquired and analyzed to accurately profile individual cells (Fig. 1a–e). Notably, ~82% of SMCs within atherosclerotic lesions (YFP⁺ACTA2⁺) were ACTA2 negative (Fig. 1a and Supplementary Table 1), indicating that the majority of SMCs within the lesion cannot be identified using traditional SMC markers. These results also showed that phenotypically modulated SMCs (YFP⁺ACTA2⁻) comprised approximately 30% of the total cells within lesions (Fig. 1a and Supplementary Table 1), which far exceeds previous estimates based on ACTA2 immunostaining.

SMCs within atherosclerotic plaques express markers of macrophages, MSCs, and myofibroblasts

We found that phenotypically modulated (YFP⁺) SMCs within lesions expressed markers of macrophages (LGALS3) (Fig. 1c), MSCs (SCA1) (Fig. 1d), and myofibroblasts (ACTA2 and PDGFB) (Fig. 1e). From these data we estimate the following distribution of SMC-derived cells within lesions: 30% macrophage-like cells (YFP⁺ACTA2⁻LGALS3⁺), 7% MSC-like cells (YFP⁺ACTA2⁻SCA1⁺), 12% myofibroblast-like cells (YFP⁺ACTA2⁺PDGFB⁺), and 32–51% an indeterminate cell phenotype (YFP⁺ACTA2⁻LGALS3⁻SCA1⁻) (Supplementary Table 1). In addition, these analyses showed that 36% of LGALS3⁺ cells within advanced atherosclerotic lesions were YFP⁺, indicating that approximately one-third of cells that would normally be classified as macrophages in most previous studies in the field actually originated from SMCs, rather than from myeloid cells as previously assumed. These initial studies were performed in paraffin-embedded samples to maintain the ultrastructure of the plaque and to determine the location of various SMC-derived cells within lesions. However, this technique limits the number of markers that can be simultaneously examined. To further characterize SMC-derived plaque cells, we performed flow cytometric analyses of freshly dissociated cells from the aorta, from the aortic root through the iliac bifurcation. We found that substantial numbers of SMC-derived cells expressed multiple additional macrophage and hematopoietic markers (Fig. 1f). In particular, we identified YFP⁺ cells that co-expressed the monocye/macrophage marker ITGAM (CD11b) and the mature macrophage marker F4/80, as well as YFP⁺ cells that co-expressed ITGAM and the dendritic cell marker ITGAX (CD11c). In addition, we found that 13% of the YFP⁺ cells co-expressed the MSC markers SCA1 and ENG (CD105) (Fig. 1f). When we analyzed MSC-like cells using traditional negative-gating strategies (CD45⁻CD34⁻CDH5⁻), we found that up to 45% of MSC-like cells within the aortas of 18-week Western diet–fed SMC YFP⁺/⁻ Apc⁻/⁻ mice were YFP⁺ (Supplementary Fig. 3a), although it is unclear whether all of these cells were located within lesions or whether they also contributed to...
the population of adventitial SCA1+ cells that have previously been described by several groups28–32. Gating strategies for all flow cytometry experiments were determined on the basis of fluorescence minus one (FMO) controls (Supplementary Fig. 3b,c).

To further assess the morphological and possible functional properties of SMC-derived macrophage-like cells in vivo, we analyzed BCA lesions from SMC YFP+/Apoe−/− mice with transmission electron microscopy, combined with detection of YFP expression by immunogold labeling. As shown in Figure 2a and Supplementary Figure 4, we identified YFP+ cells containing multiple large lipid vacuoles that seem to be phagocytic. However, these cells were relatively rare, possibly reflecting the low frequency of phagocytic YFP+ cells at any given instant in time, the transient nature of this process, and/or technical difficulties in detecting these cells by immunoelectron microscopy. In addition, we flow-sorted SMC-derived MSC-like cells (YFP+/ENG−SCA1+), non-SMC-derived MSC-like cells (YFP+/ENG+SCA1+), and SMC-derived non-MSC-like cells (YFP−/ENG+SCA1−) from 18-week Western diet–fed lineage-tracing mice to test the ability of these cell populations to differentiate into multiple lineages, including adipocytes and osteoblasts. After two passages in MSC maintenance medium, the SMC non-MSC-like cells became unhealthy in appearance and died (data not shown). The SMC-derived MSC-like cells survived but seemed to be senescent (Fig. 2b) and grew slowly (data not shown). These cells also failed to differentiate into either adipocytes (Fig. 2c,d) or osteoblasts (data not shown) when exposed to the appropriate differentiation culture medium. In contrast, the YFP+ (non-SMC-derived) MSC-like cells grew well (data not shown) and showed high-efficiency differentiation into adipocytes (Fig. 2c,d) and osteoblasts (data not shown). These data indicate that although a subset of SMC-derived cells within atherosclerotic lesions express multiple markers of MSCs, these cells do not appear to be pluripotent and thus may not have the functional properties of MSCs.

**SMCs within human atheromas express the macrophage marker CD68**

To independently detect phenotypically modulated SMCs that express LGALS3 and to validate a method for detecting these cells in human lesions, we used an in situ hybridization proximity ligation assay (ISH-PLA) recently developed by our laboratory27. This technique permits the identification of phenotypically modulated SMCs within fixed tissues based on the detection of the histone H3K4DiMe in the Myh11 promoter (PLA+), a SMC-specific epigenetic signature that persists in cells that have no detectable expression of SMC markers27,33. We first validated this method by showing that YFP+LGALS3+ SMCs within the lineage-tracing mice retained this SMC-specific epigenetic signature (Supplementary Fig. 5a). We also showed that neither cultured RAW 264-7 mouse macrophage cells (Supplementary Fig. 5b) nor human monocytes (Supplementary Fig. 5c) exposed to POVP, an oxidative product of LDL that activates monocytes/macrophages34, exhibited H3K4diMe in the Myh11 promoter.

To determine whether SMC transition to a macrophage-like state occurs in human lesions, we stained human coronary artery atherosclerotic lesions, we used an in situ hybridization proximity ligation assay (ISH-PLA) recently developed by our laboratory27. This technique permits the identification of phenotypically modulated SMCs within fixed tissues based on the detection of the histone H3K4DiMe in the Myh11 promoter (PLA+), a SMC-specific epigenetic signature that persists in cells that have no detectable expression of SMC markers27,33. We first validated this method by showing that YFP+LGALS3+ SMCs within the lineage-tracing mice retained this SMC-specific epigenetic signature (Supplementary Fig. 5a). We also showed that neither cultured RAW 264-7 mouse macrophage cells (Supplementary Fig. 5b) nor human monocytes (Supplementary Fig. 5c) exposed to POVP, an oxidative product of LDL that activates monocytes/macrophages34, exhibited H3K4diMe in the Myh11 promoter.

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We found MYH11 H3K4diMe PLA+CD68+ cells that were Y chromosome-negative (Fig. 3d), consistent with the notion that these macrophage-like cells are of SMC and not hematopoietic origin. Notably, we never found MYH11 H3K4diMe PLA+ cells that were Y chromosome-positive (Fig. 3d and data not shown), demonstrating that myeloid cells do not acquire the MYH11 H3K4diMe SMC epigenetic signature in human atherosclerotic lesions.

KLF4 has a critical role in regulating SMC phenotype and plaque pathogenesis

We have previously shown that KLF4, a cell pluripotency factor35, is required for SMC phenotypic switching in several in vitro models25,36. However, there is as yet no evidence that SMC phenotypic transitions within atherosclerotic lesions are KLF4 dependent, and, if so, what role these transitions have in lesion pathogenesis. Consistent with our hypothesis that KLF4 regulates phenotypic transitions of SMCs within atherosclerotic lesions, we observed large numbers of YFP+ cells that expressed KLF4 within BCA lesions from 18-week Western diet–fed SMC YFP+/+ ApoE−/− mice (Supplementary Fig. 7a). To determine whether KLF4 regulates SMC phenotypic transitions and overall lesion pathogenesis, we generated SMC YFP+/+ ApoE−/− mice with SMC-specific deficiency of KLF4 (SMC YFP+/+ Klf4Δ/sApoE−/− mice) by crossing SMC YFP+/+ ApoE−/− mice with Klf4Δ/s mice. We observed a high level of recombination of the floxed Klf4 alleles (Klf4Δ/s) tamoxifen treatment (Supplementary Fig. 7b,c), including what we estimate to be nearly 100% recombination within SMCs in the aorta when the data are corrected for the >40% of non-SMC DNA present in these samples, based on flow cytometric analysis of aortic cell populations (Supplementary Fig. 2b). Figure 4a shows representative confocal immunofluorescence images of the BCA lesions of control mice (SMC YFP+/+ Klf4+/+ ApoE−/−) and mice with loss of KLF4 in SMCs (SMC YFP+/+ Klf4Δ/sApoE−/−) after being fed a Western diet for 18 weeks. SMC YFP+/+ Klf4Δ/sApoE−/− mice had a nearly 50% reduction in plaque size (Fig. 4b) and multiple changes consistent with increased plaque stability, including a more-than-twofold increase in fibrous cap area (Fig. 4c), an increase in the percentage of ACTA2+ cells within the fibrous cap (Fig. 4d), and a reduced percentage of LGALS3+ cells (Fig. 4e) as compared to control SMC YFP+/+ Klf4+/+ ApoE−/− mice.

SMC lineage-tracing analyses showed that loss of KLF4 within SMCs did not result in a change in the overall number of SMCs (YFP+ cells) within the lesions (Supplementary Fig. 8a), but it had major effects on SMC phenotypic transitions. These effects included a 53% decrease in the percentage of macrophage-like SMCs (YFP+ LGALS3+/YFP+) within a lesion (Fig. 4e), a 70% decrease in the percentage of MSC-like SMCs (YFP+ SCA1+/YFP+) within the medial area underlying lesions (Supplementary Fig. 8b), but no change in the percentage of MSC-like SMCs in the lesion itself (Supplementary Fig. 8c) in SMC YFP+/+ Klf4Δ/s ApoE−/− mice as compared to control SMC YFP+/+ Klf4+/+ ApoE−/− mice. Consistent with these results, flow cytometric analyses showed a decrease in the percentage of SMC-derived MSC-like cells (YFP+ SCA1+/YFP+ CD68− FABP4+) in vivo (Supplementary Fig. 8d), but no change in either the overall percentage of MSCs (Supplementary Fig. 8e) or the percentage of YFP+ cells (Supplementary Fig. 8f) in SMC YFP+/+ Klf4Δ/s APOE−/− mice as compared to control SMC YFP+/+ Klf4+/+ ApoE−/− mice. As noted above, SMC specific Klf4 knockout mice showed an increase in the percentage of ACTA2+ cells within the fibrous cap (Fig. 4d) as compared to wild-type control mice. Similarly, SMC YFP+/+ Klf4Δ/s APOE−/− mice showed an increase in the percentage of ACTA2+ cells within lesions (Fig. 4f), but reduced proliferation of SMC-derived cells (Fig. 4g) and a marked reduction in YFP+ SMC apoptosis as compared to control YFP+/+ Klf4+/+ ApoE−/− mice (Fig. 4h). These effects were not associated with changes in medial or luminal area (Supplementary Fig. 8g), or in the percentage of cells that were YFP+ PDGFβR+ (Supplementary Fig. 8h) or YFP+ ACTA2+ (Fig. 4f). In addition, we did not observe changes in cholesterol or triglyceride levels (Supplementary Fig. 8i).

KLF4 modulates phenotypic transitions and functional properties of SMCs

We have previously presented evidence that Klf4 is induced in cultured SMCs by treatment with oxidized phospholipids36 and that KLF4 suppresses expression of SMC marker genes through several mechanisms, including binding to the G/C repressor element found in most SMC marker gene promoters (including Acta2, Tagln, and Myh11) and inhibiting binding of the transcription factor SRF to CARG elements16,37,38. To determine whether similar mechanisms contribute to suppression of SMC marker gene expression within atherosclerotic lesions in vivo, we performed chromatin immunoprecipitation (ChIP) assays on chromatin extracted from the BCA regions of ApoE−/− mice carrying a transgene containing either an
unmutated Tagln promoter driving lacZ expression, or a Tagln promoter with a mutation of the GC repressor element driving lacZ expression. We found that compared to chow-fed Apoe/− mice, Western diet–fed Apoe/− mice showed marked enrichment of KLF4 binding to the Acta2, Tagln, Myh11, and Cnn1 endogenous promoters (Fig. 5a). Using the two transgenic strains described above, we showed that enhanced KLF4 binding to the Tagln promoter under Western diet conditions was dependent on the G/C repressor element (Fig. 5b). Finally, using the ISH-PLA assay, we showed that KLF4 bound to the Tagln promoter in individual phenotypically modulated SMCs (YFP+ACTA2− cells) within lesions of Apoe/− mice (Fig. 5c). Taken together, results provide compelling evidence that coordinated suppression of SMC marker gene expression is mediated by direct binding of KLF4 to the promoters of SMC marker genes.

We21,23 and others39–42 have shown that KLF4 can act as either a transcriptional repressor or activator, depending on the cell type and gene locus. To more fully define the repertoire of KLF4 target genes that mediate SMC phenotypic switching, we performed KLF4 ChIP-seq analysis on chromatin samples derived from BCA isolated from SMC YFP+/+Klf4+/+Apoe−/− versus SMC YFP+/+Klf4Δ/ΔApoe−/− mice. This analysis required pooling of BCA samples from 16 mice per group to obtain sufficient DNA. Notably, we identified 869 KLF4 target genes that were selectively enriched in Klf4+/+Apoe−/− versus Klf4Δ/ΔApoe−/− Western diet–fed mice (Fig. 5d and Supplementary Table 2). These putative KLF4 target genes that were selectively bound by KLF4 in SMCs include the SMC marker genes Acta2 and Tagln, thereby validating the fidelity of our ChIP-seq analysis. In addition, we found evidence of enriched KLF4 binding in genes within regulatory pathways that are likely to be important in the pathogenesis of lesions, including gene families associated with phagocytosis, apoptosis, cell migration, and inflammation (Fig. 5d and Supplementary Table 2). These putative KLF4 target genes probably contributed to the beneficial effects of SMC-specific loss of KLF4 on lesion size and plaque pathogenesis. KLF4 also bound regions near Itgal (CD11a), Itgax (CD11b), Itgam (CD11c) (Fig. 5d and Supplementary Table 2), and Arg1 (data not shown) in Klf4+/+ but not Klf4Δ/Δ mice. We also noted 459 KLF4 targets enriched in Klf4Δ/ΔApoe−/− samples as compared to Klf4+/+Apoe−/− samples (Supplementary Fig. 9 and Supplementary Table 3), presumably representing KLF4 target genes in non-SMCs that were altered as a secondary consequence of the loss of KLF4 in SMCs.

Figure 3 SMCs within human coronary artery lesions express the macrophage marker CD68. SMCs within advanced atherosclerotic lesion specimens were identified by PLA detection of the SMC-specific stable epigenetic signature H3K4diMe in the MYH11 promoter. MYH11 H3K4diMe PLA+ cells exhibit a punctate red dot within the nucleus; the non-nuclear amorphous red staining is autofluorescence or nonspecific background. (a) Samples treated for PLA (red) were immunostained for CD68 (green), and DAPI (blue). (b) Samples treated for PLA (red) were immunostained for ACTA2 (green), CD68 (cyan) and DAPI (blue). Shoulder regions within plaques showed a high incidence of SMC-derived macrophage-like cells (PLA+/CD68−) (yellow arrows). Phenotypically modulated SMCs negative for CD68 (PLA−CD68+) were also observed (white arrows). Scale bars, 50 μm. (c) Combined epigenetic SMC and genetic HSC lineage tracing analysis of a cross-gender human heart transplant sample. Coronary artery specimens from a male patient who had received a female heart were processed for PLA (red), Y chromosome (Y-Chr) FISH (green), and CD68 staining (yellow). A PLA−Y chromosome−CD68− cell (yellow arrows) represents a macrophage of hematopoietic origin. A large number of cells of hematopoietic origin that are negative for CD68 (PLA−Y chromosome−CD68−) were also observed (white arrows). Scale bars, 50 μm.
Given our observation that SMC-specific loss of Klf4 is associated with a marked reduction in the percentage of SMC-derived macrophage-like cells in Klf4<sup>fl/fl</sup> mice as compared to wild-type Klf4<sup>+/+</sup> control mice (Fig. 4f), we tested whether KLF4 is required for transition of cultured SMCs to a macrophage-like state in vitro following cholesterol loading<sup>12</sup>. Owing to recent controversies concerning the origin and purity of SMCs cultured from mouse vessels<sup>43,44</sup>, we used SMCs obtained by sorting freshly isolated YFP<sup>+</sup> aortic cells from our SMC lineage-tracing mice. Primary cultures of aortic SMCs harvested from 9-week-old tamoxifen-injected SMC YFP<sup>+/+</sup> Apoe<sup>−/−</sup> mice that had been injected with tamoxifen between 6–8 weeks of age were >98% YFP<sup>+</sup> (Supplementary Fig. 10a), indicating that these cultured SMCs are derived from mature differentiated SMCs in vivo and not from a stem cell source, as has been speculated<sup>43,44</sup>. Cholesterol loading of cultured SMCs resulted in increased expression of Lgals3 (Fig. 6a) and Klf4 (Supplementary Fig. 10b) as compared to vehicle-loaded control cells; the increase in Lgals3 expression was abolished in aortic SMCs derived from SMC YFP<sup>+/+</sup> Klf4<sup>Δ/Δ</sup> mice (Fig. 6a). Cholesterol loading was also associated with increased phagocytic behavior of the cells as compared to vehicle-loaded control cells that was KLF4 dependent (Fig. 6b,c).

Finally, cholesterol loading resulted in KLF4-dependent increases in the expression of the pro-inflammatory cytokines MCP1, CXCR1, STNFR1 (Supplementary Fig. 10c–e), Lgals3 mRNA (Fig. 6a), and the MSC markers Sca1 and Eng (Supplementary Fig. 10f,g) as compared to vehicle-loaded control cells. In contrast, the increase in Abca1 mRNA expression caused by cholesterol loading was not KLF4 dependent (Supplementary Fig. 10h).

**Global heterozygous knockout of Klf4 alters plaque pathogenesis**

Previous studies using VE-cadherin-Cre Klf4<sup>B+Apoe<sup>−/−</sup></sup> and LysM<sup>Cre</sup> Klf4<sup>B+Apoe<sup>−/−</sup></sup> mouse models provided evidence that KLF4 has an atheroprotective role in endothelial cells and myeloid cells, respectively; KLF4 deficiency in these strains resulted in increased lesion size and changes consistent with enhanced inflammation<sup>41,42</sup>. Indeed, we confirmed the effects of myeloid-specific KLF4 deficiency by showing that LysM-Cre-dependent knockout of Klf4 was associated with increased lesion size (Supplementary Fig. 11a) and Sudan IV lipid staining (Supplementary Fig. 11b) in LysM<sup>Cre</sup> ROSA STOP floxed eYFP Klf4<sup>−/−</sup> Apoe<sup>−/−</sup> mice. However, unlike previous studies<sup>41,42</sup>, we did not observe changes in triglyceride or total cholesterol levels (Supplementary Fig. 11c). We also found a reduced number of lesional LGALS3<sup>+</sup> cells derived from LysM-Cre—expressing cells in LysM<sup>Cre</sup> Klf4<sup>−/−</sup> Apoe<sup>−/−</sup> mice as compared to wild-type control LysM<sup>Cre</sup> Klf4<sup>+/+</sup> Apoe<sup>−/−</sup> mice, based on YFP expression (Supplementary Fig. 11d). However, these results are equivocal regarding the identity of the YFP<sup>+</sup> cell types affected by KLF4 deficiency because cells other than myeloid cells, including SMCs, may express the LysM-Cre transgene.
Because the loss of Klf4 in SMCs had opposite effects on plaques to Klf4 loss in endothelial or myeloid cells, a key unresolved question is whether global conditional loss of Klf4 would be beneficial or detrimental. To test this, we generated tamoxifen-inducible homozygous Klf4-knockout mice (ERT²-Cre⁺ Klf4fl/flApoe−/−) and heterozygous Klf4-knockout mice (ERT²-Cre⁺ Klf4fl/+Apoe−/−); the latter represent a model of partial inhibition of KLF4 across all cell types and thus mimic potential therapeutic approaches that would achieve partial suppression of KLF4. Unfortunately, conditional global homozygous Klf4 mice had to be euthanized at 8–10 weeks after Western diet feeding owing to excessive weight loss and the development of skin lesions; these effects are presumably due to the role of KLF4 in regulating the proliferation and differentiation of epithelial cells. Tamoxifen-treated ERT²-Cre⁺ Klf4fl/+Apoe−/− mice (ERT²-Cre⁺ Klf4fl/+Apoe−/−) demonstrated 50% recombination (the maximum possible recombination for a heterozygous floxed allele) in the aorta, liver, and colon (Supplementary Fig. 12a,b), but exhibited no changes in body weight, heart weight, or cholesterol and triglyceride levels when compared to tamoxifen-treated ERT²-Cre⁺ Klf4fl/+Apoe−/− littermate control mice (Supplementary Fig. 12c).

Notably, mice with conditional global heterozygous Klf4 loss had similar effects with respect to atherosclerotic lesions as those observed for SMC-specific conditional Klf4 loss, including a 30% decrease in lesion size (Supplementary Fig. 13a), and signs of increased plaque stability, namely increased ACTA2+ cap coverage (Supplementary Fig. 13b) and decreased LGALS3+ area (Supplementary Fig. 13c). In addition, conditional global heterozygous Klf4-knockout mice exhibited decreased intraplaque hemorrhage (Supplementary Fig. 13d) and decreased apoptosis and cell proliferation (Supplementary Fig. 13e,f) as compared to ERT²-Cre⁺ Klf4fl/+Apoe−/− control mice. Taken together, these results indicate that global loss of one Klf4 allele has beneficial overall effects on plaque development, leading to smaller and possibly more stable lesions.

**DISCUSSION**

Despite numerous reports showing that cultured SMCs downregulate the expression of SMC differentiation marker genes after exposure to environmental cues present in atherosclerotic lesions, including cholesterol, POVPSC, PDGF-BB, and interleukin (IL)-1β (ref. 50), the field has relied almost entirely on the detection of these markers
to ascertain whether a given cell within a lesion is a SMC. Indeed, this practice has contributed to the well-established dogma in the field that the role of SMCs within plaques is rather limited, albeit presumed to be beneficial by virtue of the role of phenotypically modulated SMCs in producing extracellular matrix and thereby contributing to fibrous cap formation. Herein we show that >80% of SMCs within BCA lesions are phenotypically modulated and thus would have been undetected by conventional techniques. These cells comprise ~30% of the total cellular composition of the lesion. Moreover, we show that phenotypically modulated SMCs transition to cells with multiple phenotypes within lesions, including cells that express markers of macrophages, MSCs, and/or myofibroblasts. Most notably, we show that these transitions are functionally important in that selective loss of Klf4 within SMCs results in reduced lesion size, increased fibrous cap thickness, and major reductions in the fraction of SMC-derived macrophage- and MSC-like cells, but causes an increase in the fraction of ACTA2+ cells within the fibrous cap. In addition, we show that cholesterol loading of cultured SMCs induces KLF4-dependent activation of macrophage and MSC markers, expression of pro-inflammatory cytokines, and increased phagocytic behavior. Finally, our in vivo KLF4 ChIP-seq analyses identified >800 putative KLF4 regulated genes within SMCs, including many associated with pro-inflammatory processes. Taken together, these results provide compelling evidence that transitions in SMC phenotype have a crucial role in lesion development, plaque composition, and stability, and suggest that therapeutic approaches aimed at promoting beneficial changes in SMC phenotype may be a viable means of treating advanced atherosclerosis.

A key question is how the loss of Klf4 within SMCs results in a marked reduction in overall lesion size as well as in multiple changes consistent with increased plaque stability. Our data indicate that these effects are not due to a change in the number of SMC-derived cells within lesions. Recent studies by the Fisher laboratory found that although some SMCs express macrophage markers after cholesterol loading in vitro, principal component analysis of microarray data revealed that these cells are distinctly different from classical monocytes, macrophages, and dendritic cells. Moreover, these cells have reduced phagocytic capacity. Notably, we found that SMC derived MSC-like lesional cells seem to be dysfunctional. Accordingly, we postulate that the loss of Klf4 within SMCs results in phenotypic transitions that have favorable effects in inhibiting plaque pathogenesis, including the loss of SMC-derived cells with ‘pro-inflammatory’ macrophage-like properties, and the gain of SMC-derived cells that contribute to plaque stabilization through mechanisms and functions yet to be defined. Although it has long been postulated that SMCs within lesions have a beneficial role, as indicated in many review articles, our findings show that this is an oversimplification and that the effects of SMCs can vary dramatically depending on the nature of their phenotypic transitions. A critical challenge for future studies will be to identify the environmental cues within advanced atherosclerotic lesions that regulate phenotypic transitions of SMCs, as well as the other major cell types within lesions, and to determine how these might be manipulated therapeutically to reduce plaque burden and increase plaque stability.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. The GEO accession number for KLF4 ChIP-seq data is GSE65812.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

L.S.S. conducted experiments; performed data analysis; generated most of the experimental mice; performed immunostaining, image analysis and flow cytometry; and was primary writer of the manuscript. D.G. performed in vitro ChIP analysis and conceived and performed all the ISH-PLA experiments. M.S. generated Tg(α2u LacZ)Apoe−/− mice, performed in vivo ChIP assays, and performed immunohistochemistry and data analysis. A.C.S. was involved in designing experiments and data analysis; assisted in image analysis, cell culture and animal experiments throughout the project. A.C.S. and B.I. participated in designing the immuno-transmission electron microscopy protocol and analysis of images. R.M.H. performed the MSC immunostaining and image analysis, and the Klf4 ChIP-seq; conducted MSC differentiation experiments; and helped design cartoons. G.F.A. conducted data analysis of ChIP-seq and the Klf4 ChIP-seq; conducted MSC differentiation experiments; and had a major role in experimental design, data interpretation, and writing the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Animal protocols were approved by the University of Virginia Animal Care and Use Committee. Male Myh11-CreERT2 (ref. 54), LysoM2-GFP, Apoe−/−, ROSA26 STOP-fox eYFP+/−, Klf4−/− (ref. 47), and ERT-Cre (ref. 55) mice were used in this study. We also used male Apoe−/− transgenic mice carrying either an unmutated Tagln promoter-lacZ transgene, or a G/C repressor-mutated version of the Tagln promoter-lacZ transgene13. ROSA26 STOP-fox eYFP+/− and Apoe−/− mice were obtained from Jackson Laboratories. Myh11-CreERT2, ROSA26 STOP-fox eYFP, Klf4−/− and ERT-Cre mice were genotyped by PCR as previously described27,47,54,55. For both the Myh11-CreERT2 and ERT-Cre mouse models, Cre recombines was activated in male mice with a series of ten 1-mg tamoxifen (Sigma, cat. no. T-5648) intraperitoneal injections from 6 to 8 weeks of age, for a total of 10 mg of tamoxifen per mouse, which averaged 25 g of body weight during this 2-week period. Male littermate controls were used for all studies. Mice were fed a Western diet containing 21% milk fat and 0.15% cholesterol (Western diet, Harlan Teklad) for 18 weeks starting at the end of tamoxifen treatment. Irradiated mouse standard chow diet was purchased through Harlan (cat. no. 7012). Mice were euthanized by CO2 asphyxiation and then perfused via the left ventricle as follows: 5 ml PBS, 10 ml 4% paraformaldehyde, 5 ml PBS. Brachiocephalic arteries were carefully dissected and fixed for an additional 4% paraformaldehyde before they were embedded in paraffin with the exception of SCA1-stained tissues, which were snap-frozen in Richard-Allan Scientific Neg-50 Frozen Section Medium (Thermo Scientific) before sectioning. Assays for determining total plasma cholesterol and triglyceride levels (Abbott). Mice were allocated to experimental groups based on genotyping and then randomized for the various experimental measurements. Any animal with triglyceride or cholesterol levels beyond 3 s.d. of the mean level for all mice had received a heart from a female donor (n = 1). The Institutional Review Board at University of Virginia approved the use of all autopsy specimens.

Analysis of atherosclerotic plaques. Paraffin-embedded brachiocephalic arteries (BCAs) were serially sectioned at a 10-µm thickness from the aortic arch to the right subclavian artery. For immunofluorescence analysis of LgalS3 SMCs within the BCA, three sections were analyzed from a location 150 µm, 450 µm, and 750 µm from the start of the branch of the BCA from the aortic arch. Slides were stained with antibodies specific to GFP (Abcam ab6673), ACTA2 (Sigma F3777), LGALS3 (Cedarlane CL8942AP), MK67 (Abcam ab15580), CASP3 (Cell Signaling 9661S), KLFR4 (R&D Systems AF3158), MYH11 (Kamiya Biomedical Company MC-352), PDGFBR (Abcam ab52370), and SCA1 (Ly6A/E) (Abcam ab51317). Using a Zeiss LSM700 confocal microscope, a series of eight z-stack images of 1 µm in thickness were acquired for further analysis. Owing to variation in cellular composition in different regions of the lesion, cells from five standardized fields (two near the shoulder, one near the fibrous cap, and two near the media), each 14,283 µm² in area, were counted to determine the cellular composition within each lesion. Close examination of each plane of the z-stack was conducted using Zen 2009 Light Edition Software (Zeiss) to ensure the presence of immunofluorescence staining coinciding with a single DAPI+ nucleus. The region of the lesion within 30 µm of the luminal boundary, as determined using Zen 2009 Light Edition Software, was analyzed to determine the cellular composition of the lesion cap, and the area of this region was compared to the entire area of the atherosclerotic lesion to determine cap area/lesion area. Morphometric analyses of lesion size were completed using ImagePro Plus (Media Cybernetics) as described in Alexander et al.56. Researchers were blinded to the genotype of the animals until the end of the analysis.

Immuno-transmission electron microscopy. SMC YFP+/+ Apoe−/− mice fed a Western diet for 18 weeks were euthanized by CO2 asphyxiation and perfusion fixed with 0.3% glutaraldehyde (Electron Microscopy Sciences 16300), 4% paraformaldehyde (Electron Microscopy Sciences 15700) in 1× PBS. Brachiocephalic arteries were isolated, frozen in liquid nitrogen, and sent to the University of Virginia Advanced Microscopy Core for processing. Grids were stained with an antibody specific to GFP (Abcam ab6673), followed by staining with a rabbit anti-goat secondary conjugated to 10-nm gold beads (Electron Microscopy Sciences 25229). Images were captured using a FEOL 1230 transmission electron microscope with an ultra-high resolution capture camera (Gatan UltraScan 1000 2k x 2k CCD digital imaging camera).

Flow cytometry. SMC YFP+/+ Apoe−/− mice were euthanized by CO2 asphyxiation after 18 weeks of Western diet treatment. Mice were then perfused with 10 ml of PBS, and the aorta from the iliac bifurcation to the aortic root was gently cleaned of fat and fascia before removal from the animal. After removal from the animal, the heart was dissected away from the aortic root and the tissue was placed into an enzyme cocktail containing 4 U/ml Liberase TM (Roche 0540119001), 0.1 mg/ml DNase I, and 60 µg/ml hyaluronidase in RPMI-1640. Once immersed in the digestion cocktail, the tissue was cut longitudinally, minced, and placed in a 37 °C incubator for 1.5 h. Cells were run through a 70-µm strainer and spun down at 500g for 5 min. Cells were resuspended in red blood cell lysis buffer (BD PharmLyse 555899) for two minutes and then inactivated using serum containing media, spun down again, and resuspended in 200 µl of 1× PBS. SMC-derived macrophage-like cells were identified using antibodies specific to F4/80 (eBioscience 17-4801), FPRC (eBioscience 47-0451), ITGAM (eBioscience 45-0121), DAPI (Innogenetix), LGALS3 (BioLegend 125405), and ITGAX (eBioscience 25-0114). SMC-derived MSC-like cells were identified using negative gating for FPRC (eBioscience 12-0451-82), CDH5 (eBioscience 17-1441), and CD34 (BioLegend 128611), and using positive gating for ENG (eBioscience 48-1051-82) and SCA1 (eBioscience 45-5981-82). All samples were run on a Beckman Coulter CyAn ADP LX flow cytometer equipped with 405-nm, 488-nm, and 633-nm lasers.

Human specimens. De-identified atherosclerotic coronary artery specimens from patients (n = 12) were collected during autopsy. These specimens were processed, fixed in formaldehyde, and paraffin-embedded blocks were cut into 5-µm sections. One coronary artery specimen was from a male patient who received a heart from a female donor (n = 1). The Institutional Review Board at University of Virginia approved the use of all autopsy specimens.

In situ hybridization proximity ligation assay (ISH-PLA). ISH-PLA was performed as previously described27. Briefly, human MYH11, mouse Myh11, and mouse Tagln probes were generated by nick translation (using Nick Translation kit no. 10976776001, Roche) using biotin-14-dATP (Innogenetix). Biotin labeled probes (40 ng per slide) underwent denaturation in hybridization buffer (2× SSC, 50% high-grade formalamide, 10% dextran sulfate, 1 µg of human or mouse Cot-1 DNA) for 5 min at 80 °C. First, sections were stained for ACTA2 (Sigma F3777) and CD68 (Santa Cruz sc20060 KPI clone) or LGALS3 (Cedarlane CL8942AP) (Fig. 3 and Supplementary Fig. 5) or GFP (Abcam ab6673) and ACTA2 (Sigma F3777) (Fig. 5). Briefly, slides were de-paraffinized and rehydrated in a xylene and ethanol series. After antigen retrieval (Vector no. H-3300), sections were blocked with fish skin gelatin oil in PBS (6 grams per liter) containing 10% horse serum for 1 h at room temperature (21 °C). Slides were incubated with primary antibodies for 1 h at room temperature, followed by incubation with donkey anti-mouse antibody conjugated with Alexa Fluor 647 (4 µg/ml Invitrogen). Slides were then rehydrated in an ethanol series and incubated in 1mM EDTA (pH 8.0) for 20 min. Then, samples were incubated with pepsin (0.5%) in buffer (0.05 M Tris, 2 mM CaCl2, 0.01 M EDTA, 0.01 M NaCl) at 37 °C for 20 min, as previously described14. The hybridization mixture containing biotin-labeled probes (mMyh11, hMYH11 or mTagln) or a 5-TAMRA-dUTP-labeled y chromosome probe (clone RP11-88FS4, Empire Genomics) was applied on the sections. Sections were then incubated at 80 °C for 5 min, followed by 16–24–h incubation at 37 °C. Hybridization was followed by multiple washes in 2x SSC, 0.1% NP-40 buffer. PLA was performed directly after ISH according to the manufacturer’s instructions (Olink) and as previously described27. Sections were incubated blocking mouse H3K4dime (5 µg/ml, clone CMA303 Millipore) and rabbit biotin (5 µg/ml, no. ab53494, Abcam) antibodies (Fig. 3 and Supplementary Fig. 5) or mouse KLFR4 (56CT5.1.6) (2.5 µg/ml, no. ab75486, Abcam) and rabbit biotin (5 µg/ml, no. ab53494, Abcam) antibodies (Fig. 5) overnight at 4 °C. The PLA amplification step was performed using the Duolink detection kit (emission wavelength: 555 nm). Finally, mounting medium with DAPI was used to coverslip the slides. Images were acquired with an Olympus BX41 microscope fitted with a Q imaging Retiga 2000R camera. Image acquisition was performed using Q Capture Pro software (Media Cybernetics & Q Imaging, Inc.). Settings were fixed at the beginning of both acquisition and analysis steps and were unchanged. Brightness and contrast were equally adjusted after merging. Image analysis was performed with ImageJ software. To estimate the percentage of SMC-derived
macrophage-like cells, we counted the number of CD68^+PLA^+ cells in CD68 positive staining areas of human coronary lesions (n = 12). We previously estimated that the efficiency of the ISH-PLA method in mouse and human tissues is 65% (ref. 27). Considering the incomplete efficiency of ISH-PLA, we applied a correction to the percentage of CD68^+PLA^+ cells to total CD68^+ cells identified in human lesions. Both uncorrected and corrected percentages of CD68^+PLA^+ cells to total CD68^+ cells are shown in Figure 3. The corrected values were proportionally calculated by dividing the uncorrected values by 0.65, the efficiency of the in situ hybridization portion of the PLA method (see Gomez et al.27).

**ChIP assays.** Cell culture ChIP was performed as previously described27. Cells were fixed with 1% paraformaldehyde for 10 min at room temperature. Cross-linked chromatin was sheared by sonication into fragments of 200–600 base pairs. The sheared chromatin was immunoprecipitated with 2 μg of anti-H3K4me1 (clone CMA303, Millipore), or anti-KLF4 (Santa Cruz sc20691); negative control samples were incubated with mouse or rabbit IgG (Jackson Immunoresearch Laboratories). Immune complexes were captured with magnetic bead-coupled protein G (Millipore). After elution and purification of the genomic DNA (gDNA), real-time PCR was performed on immunoprecipitated (IP) and non-immunoprecipitated (INPUT) gDNA. Primer sets used for the Myh11, Arg1 and Cox2 promoters were as previously described27,40. Results are expressed as the ratio of IP/INPUT. In vivo KLF4 ChIP assays were performed as previously described27,37 using flash-frozen BCAs from mice that had been fed either 18 weeks of high-fat or standard chow (Harlan 7012) diets beginning at 8 weeks of age.

**KLF4 chromatin immunoprecipitation– sequencing (ChIP-seq).** Segments of the aorta from the arch to the aortic root and up to the carotid bifurcation isolated from 18 week western diet fed SMC Klf4^+/+YFP^+/Apoe^-/- (n = 16), and SMC Klf4^Δ/ΔYFP^+/Apoe^-/- (n = 16) mice were snap-frozen in liquid nitrogen and then processed as indicated above for ChIP assays using the KLF4-specific antibody. DNA library preparation and deep sequencing was performed by HudsonAlpha Institute for Biotechnology using the Illumina TruSeq Chip Library Kit according to the manufacturer’s protocol. Quality control and quantification of DNA and library were performed using an Agilent 2100 Bioanalyzer and a Kapa Library Quantification Kit (Kapa Biosystems) according to the manufacturer’s protocol.

**ChIP-seq data processing.** Sequencing reads from an Illumina HiSeq Sequencing System were aligned to the mouse genome (mm10) using the Bowtie alignment tool29. These aligned reads were then processed and converted into bam/bai format (http://genome.ucsc.edu/goldenPath/help/bam.html), and then loaded in the Integrative Genomics Viewer (http://www.broadinstitute.org/igv/) for visualization. The processing steps involved removing duplicate reads and format conversions using the SAMtools29 suite. The reads were also converted to BED format (http://genome.ucsc.edu/FAQ/FAQformat#format1) for further data analysis processes such as peak calling. KLF4 peaks were identified using MACS4 (ref. 60) with ChIP-seq BED files as input files and default settings with a P value for significant peak calling ≤ 1 × 10^{-5}. Once peaks were obtained for both ChIP-seq data sets (SMC Klf4^+/+YFP^+/Apoe^-/- and SMC Klf4^Δ/ΔYFP^+/Apoe^-/-), BEDTools41 was used to remove peaks that were present in both data sets. Furthermore, BEDTools was used to identify the closest genes to each peak. If a gene was present in both data sets, the gene was removed from the analysis. Functional annotation was performed using PANTHER26 and Gene Ontology Consortium (http://geneontology.org/) software; a statistical over-representation test was performed using PANTHER26 (http://pantherdb.org/). The GEO accession number for these data is GSE65812.

**Cell culture studies.** Primary mouse aortic SMCs were isolated using a previously described protocol38 from 8-week-old SMC-lineage tracing mice after a series of ten tamoxifen injections. Cells isolated from SMC YFP^+/Klf4^+/+ and SMC YFP^+/Klf4^Δ/Δ mice were passaged three times before undergoing cell sorting for YFP^+ cells using a FACSvantage SE DIVA (Becton Dickinson). Cholesterol assays were performed using water-soluble cholesterol from Sigma (C9451-30MG) as previously published38 with a minor modification: cholesterol was reconstituted in DMEM-F12 media (Gibco) containing 0.2% FBS (Gibco), 100 U/ml penicillin/streptomycin (Gibco) and 1.6 mmol/liter l-glutamine (Gibco). Cells were allowed to grow to ~70% confluence in DF10 (DMEM-F12 medium (Gibco) containing 10% FBS (Gibco), 100 U/ml penicillin/streptomycin (Gibco) and 1.6 mmol/liter l-glutamine (Gibco)) before being switched to 0, 20, 40, or 80 μg/ml cholesterol-containing medium. After 72 h, cells were harvested for miRNA, protein, or ChIP analysis. Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) according to the respective manufacturer’s instructions. One microgram of RNA was used to perform a reverse transcription with iScript cDNA Synthesis Kit (Bio-Rad). A SensiFAST SYBR NO-ROX Mix (BioLine) was used to carry out RT-PCR on a C1000 Thermal Cycler CFX96TM (Bio-Rad) (Supplementary Table 4). Cell culture medium from 72 h cholesterol-treated Klf4^+/+ or Klf4^Δ/Δ cells was filtered through 0.22-μm Millex low-protein-binding Durapore filters (Merck/Millipore) to remove traces of cholesterol. Then medium was concentrated ten times using Amicon Ultra centrifugal filter devices (Millipore). Cellular proteins for cytokine assay were isolated using the RIPA buffer protocol. Briefly, cells were incubated in RIPA buffer (Pierce) with Halt Protease Inhibitor cocktail (Thermo Scientific) at 4 °C for 15 min, centrifuged, and supernatants were used for analysis. Protein concentrations were measured using the Bio-Rad DC Protein Assay Kit. Human coronary SMCs were purchased from Lonza and cultured in SMCC maintaining media (Lonza). RAW264.7 mouse macrophages (American Type Culture Collection) and human monocytes (American Type Culture Collection) were cultured per the company’s recommendations.

**Bead uptake assays.** Primary mouse aortic SMCs were treated with cholesterol as indicated above. After 72 h of cholesterol treatment, cells were switched back to DF10 culture medium containing 1.5% vol/vol 0.84-μm polystyrene beads (Spherotech FP-0870-2) for 1.5 h. Cells were then harvested, stained with LGLAS3 (Biologeck 125045) and run on an Amnis ImagingStreamX Mark II flow cytometer using a 60x objective. Data was analyzed using Amnis IDEAS software.

**Mesenchymal stem cell differentiation.** Cells were isolated from SMC YFP^+/+Apoe^-/- mice fed a Western diet for 12 or 18 weeks as described in the flow cytometry section. Next, cells were stained and negatively gated for lineage markers (CD34 BioLegend 128611, PTPRC eBioscience 56-0451-80, CDH5 eBioscience 17-1441-80), and positively gated for ENG (eBioscience 48-1051-82) and SCA1 (eBioscience 45-5981-82). YFP was detected by native fluorescence. Cells were then sorted into four populations (YFP^+SCA1^+ENG^+, YFP^+SCA1^+ENG^+, YFP^+SCA1^+ENG^+, and YFP^+SCA1^+ENG^+) using a Becton Dickinson Influx Cell Sorter. Cells were then placed in StemXVivo MSC medium (R&D Systems CCM004) media at a density of 30,000 cells/mm2 and were passed at 70% confluence (changing the medium every 2–3 d). YFP^+SCA1^+ENG^+ and YFP^+SCA1^+ENG^+ cells were expanded prior to plating for the differentiation experiments. The differentiation experiment was conducted using a kit from R&D Systems (mouse mesenchymal stem cell functional differentiation kit, SC010). Cells were differentiated in the appropriate media for 14 d (changing the medium every 2–3 d). The cells were then fixed and immunocytochemistry was performed using antibodies from the kit according to the protocol provided.

**Statistics.** Fisher’s exact test was used for categorical data. Two-way ANOVA with Tukey post hoc tests were used for multiple group comparisons determined to have normal distribution by the Kolmogorov-Smirnov test. For experiments in which multiple sections of the BCA were assessed, two-way ANOVAs were conducted to determine if there were statistical differences between the different regions of the BCA evaluated (i.e., 150 μm, 450 μm, and 750 μm from the branch of the BCA from the aortic arch). Because no statistical differences were seen between BCA regions, we calculated an average across all regions. Nonparametric data were analyzed using the Wilcoxon rank-sum test. There were no significant interactions between genotype and region (from the start of the BCA) for any of the endpoints analyzed by two-way ANOVA. P < 0.05 was considered significant. SAS v9.3 with Enterprise Guide v5.1 software (SAS Institute Inc.) was used for all statistical analyses.

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Corrigendum: KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis
Laura S Shankman, Delphine Gomez, Olga A Cherepanova, Morgan Salmon, Gabriel F Alencar, Ryan M Haskins, Pamela Swiatlowska, Alexandra A C Newman, Elizabeth S Greene, Adam C Straub, Brant Isakson, Gwendalyn J Randolph & Gary K Owens
Nat. Med. 21, 628–637 (2015); published online 18 May 2015; corrected after print 12 August 2015
In the version of this article initially published, the labels to the left of the two micrographs in Figure 2c are reversed. Also, in Figure 4g, MKi67, used as a cell proliferation marker, is misspelled. The errors have been corrected in the HTML and PDF versions of the article.

Corrigendum: Metformin activates a duodenal Ampk–dependent pathway to lower hepatic glucose production in rats
Frank A Duca, Clémence D Côté, Brittany A Rasmussen, Melika Zadeh-Tahmasebi, Guy A Rutter, Beatrice M Filippi & Tony K T Lam
Nat. Med. 21, 506–11 (2015); doi:10.1038/nm.3787; published online 06 April 2015; corrected after print 7 May 2015
In the version of this article initially published, we incorrectly reported the value for the particles per milliliter of Ad-dn-AMPK (D157A) used in the study. It was 3.1 × 10–9 PFU ml–1 and not 1.1 × 10–13 PFU ml–1 as originally reported. The errors have been corrected in the HTML and PDF versions of the article.

Erratum: Cardiac RKIP induces a beneficial β-adrenoceptor–dependent positive inotropy
Evelyn Schmid, Stefan Neef, Christopher Berlin, Angela Tomasovic, Katrin Kahler, Peter Nordbeck, Katharina Deiss, Sabrina Denzinger, Sebastian Herrmann, Erich Wettwer, Markus Weidendorfer, Daniel Becker, Florian Schäfer, Nicole Wagner, Suleyman Ergün, Joachim P Schmitt, Hugo A Katus, Frank Weidemann, Ursula Ravens, Christoph Maack, Lutz Hein, Georg Ertl, Oliver J Müller, Lars S Maier, Martin J Lohse & Kristina Lorenz
Nat. Med. 21, 1298–1306 (2015); published online 19 October 2015; corrected after print 29 October 2015
In the version of this article initially published, there are typographical errors in the labels to Fig. 3e–h: ’WT + AAV9-eGFP’ and ’WT + AAV9-RKIPWT’ are incorrect, and should be ’RKIP – + AAV9-eGFP’ and ’RKIP + + AAV9-RKIPWT’ respectively. The errors have been corrected in the HTML and PDF versions of the article.

Erratum: Snail1-induced partial epithelial-to-mesenchymal transition drives renal fibrosis in mice and can be targeted to reverse established disease.
M Teresa Grande, Berta Sánchez-Laorden, Cristina López-Blau, Cristina A De Frutos, Agnès Bouetot, Miguel Arévalo, R Grant Rowe, Stephen J Weiss, José M López-Novoa & M Angela Nieto
Nat. Med. 21, 989–997 (2015); published online 03 August 2015; corrected after print 4 September 2015
In the version of this article initially published, the word ‘genetically’ was added by the editor to the penultimate sentence of the abstract during proofing of the manuscript. However, the authors used not only a genetic knockout approach but also morpholino-induced inhibition of proper mRNA processing to target Snail1 expression. Thus, the word ‘genetically’ has been deleted from the sentence to better convey the findings of the report. The error has been corrected in the HTML and PDF versions of the article.

Erratum: Genomic landscape of carcinogen-induced and genetically induced mouse skin squamous cell carcinoma
Dany Nassar, Mathilde Latil, Bram Boeckx, Diether Lambrechts & Cédric Blanpain
Nat. Med. 21, 946–954 (2015); published online 13 July 2015; corrected after print 6 August 2015
In the published article, the format was listed as Article, but this is a Resource. The error has been corrected in the HTML and PDF versions of the article.