Integrin beta3 regulates clonality and fate of smooth muscle-derived atherosclerotic plaque cells

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Smooth muscle cells (SMCs) play a key role in atherogenesis. However, mechanisms regulating expansion and fate of pre-existing SMCs in atherosclerotic plaques remain poorly defined. Here we show that multiple SMC progenitors mix to form the aorta during development. In contrast, during atherogenesis, a single SMC gives rise to the smooth muscle-derived cells that initially coat the cap of atherosclerotic plaques. Subsequently, highly proliferative cap cells invade the plaque core, comprising the majority of plaque cells. Reduction of integrin β3 (Itgb3) levels in SMCs induces toll-like receptor 4 expression and thereby enhances Cd36 levels and cholesterol-induced transdifferentiation to a macrophage-like phenotype. Global Itgb3 deletion or transplantation of Itgb3−/− bone marrow results in recruitment of multiple pre-existing SMCs into plaques. Conditioned medium from Itgb3-silenced macrophages enhances SMC proliferation and migration. Together, our results suggest SMC contribution to atherogenesis is regulated by integrin β3-mediated pathways in both SMCs and bone marrow-derived cells.
Atherosclerosis is a primary cause of devastating diseases of diverse arterial beds, including the coronary arteries, cerebrovasculature, peripheral arteries, and the aorta. The atherosclerotic plaque or atheroma is the culprit lesion and consists of a lipid-laden core covered by a fibrous cap. Rupture of the plaque results in thrombosis with dire consequences, such as myocardial infarction and stroke. Smooth muscle cells (SMCs) and macrophages are key players in atherogenesis, and a reduced ratio of SMCs to macrophages is associated with an unstable plaque that is prone to rupture.

Although the role of SMCs in atherogenesis of the aorta and its major branches has recently received increased attention, many of the key processes and underlying mechanisms remain to be elucidated. An in-depth understanding of the morphogenesis of SMC layers in the normal aortic media would aid in the investigation of SMC pathogenesis in vascular disease, but little is understood regarding normal aortic development. In advanced atherosclerotic plaques of high fat-fed ApoE–/− mice, recent studies indicate pre-existing SMCs give rise to alpha-smooth muscle actin (α-SMA)+ cells and predominantly SMC marker− cells, including cells expressing macrophage markers LGALS3 and CD68.[2,5]. Furthermore, depletion of the pluripotency factor Kruppel-like factor 4 (Klf4) in SMCs attenuates the expression of LGALS3; however, underlying mechanisms are not well established. Interestingly, in mature human atherosomas, 40% of CD68+ cells are also labeled by SMA, and cholesterol loading of cultured SMCs induces downregulation of SMC markers and upregulation of macrophage markers.[8,9]. These studies attest to the importance of pre-existing SMCs in atherogenesis, but a temporospatial analysis of the dynamics of SMC fate and accumulation, which promises to yield key insights, is lacking. In addition, the clonal architecture of smooth muscle-derived cells in plaques is controversial,[2,10–15] and has major ramifications on pathogenesis and potentially on therapeutic approaches: distinct strategies may be pursued to tailor therapies to target multiple progenitors (i.e., polyclonal plaque) or a single progenitor (i.e., monoclonal plaque). The roles of non-smooth muscle cells in modulating SMC fate and clonality have not been investigated, and no molecular or cellular regulators of SMC clonal patterning have been identified.

Integrins are heterodimeric transmembrane proteins, composed of α and β subunits, that link the extracellular matrix to the cytoskeleton, and global deletion of Itgb3 (encoding integrin β3) exacerbates atherosclerosis in high fat-fed ApoE–/− mice.[16] Although mechanisms underlying this exacerbation are not well established, Itgb3−/− macrophages have been implicated.[17] In addition to hematopoietic cells (i.e., macrophages and platelets), β3 is highly expressed in SMCs, and we and other have demonstrated that β3 is critical for enhanced SMC proliferation and migration in vascular disease.[18–20] Treatment of arterial SMCs with an α5β1 antagonist attenuates fibronectin-induced SMC downregulation,[21] but the role of β3 in SMC differentiation or transdifferentiation is not known.

Herein, we use multi-color genetic labeling and single cell resolution clonal analysis to demonstrate that the normal aortic tunica media forms by mixing of multiple SMC progenitors. However, in ApoE–/− mice, a single SMC that is present prior to the onset of high fat feeding gives rise to the majority of cells in an advanced atherosclerotic plaque. Furthermore, these studies identify cap cells in the early plaque as specialized cells that derive from a single pre-existing SMC and have a unique molecular signature, expressing markers of SMCs (SMAD) smooth muscle myosin heavy chain (SMMHC)) and undifferentiated mesenchyme (platelet-derived growth factor receptor (PDGFR)-β), as well as integrin β3. In human and murine plaques, cap cells are highly proliferative, and early cap cells give rise to progeny that down-regulate SMC markers and β3, and migrate into the core.

Reduction of β3 stimulates SMC transdifferentiation by enhancing toll-like receptor 4 (Tlr4) and Cd36 expression, oxidized (ox)-LDL uptake, and cholesterol-induced downregulation of SMC differentiation markers and upregulation of macrophage markers. In addition, in the ApoE–/− background, in contrast to Itgb3 wild-type mice, global Itgb3 deletion, or transplant of Itgb3−/− bone marrow results in recruitment of multiple pre-existing SMCs into plaques. Finally, in comparison to ApoE–/− macrophage-conditioned medium, medium conditioned by ApoE−/−, Itgb3−/− macrophages induces SMC migration and proliferation. Thus, through both cell autonomous and non-cell autonomous effects, β3 regulates key factors—the number of SMCs recruited into the plaque and their fate—that dictate the course of atherosclerosis, and further investigation into regulation of these processes is needed.

Results
Multiple progenitors mix extensively to form the aortic wall.

Based on PCR analysis of X-chromosome inactivation patterns (as a crude clone marker) in micro-dissected adult human descending aorta, previous studies have suggested that the arterial wall is composed of large clonal SMC patches.[11,12] Herein, we initially focused on the development of the murine descending aorta (Supplementary Fig. 1), and used single cell resolution clonal analyses and modern genetic tools to determine whether the progeny of individual early progenitor cells is cohesive or dispersed (Fig. 1). A single high dose (150 µg) of 4-hydroxy tamoxifen (4-OH-T) in dams pregnant with embryos carrying the multi-color ROSA26R-Rainbow (Rb) Cre reporter[22] and the ubiquitous ROSA26R-CreER[23] at embryonic day (E) 5.25 (the earliest time that reproducibly yields viable embryos) results in substantial intermixing of cells of different colors (i.e., polyclonal) in the thoracic descending aortic wall in ROSA26RCreER(Rb) mice at E12.5 or during adulthood (Fig. 1a–c). Furthermore, a threshold dose (20–100 µg) of 4-OH-T at E5.25 almost always results in an embryonic or adult descending aorta with either no labeled cells or all labeled cells of a single color (Fig. 1d–g and Supplementary Table 1; Methods). Thus, these marked cells, which have a diffuse pattern with unlabeled cells interspersed, derive from a single cell present at the time of recombination (estimated to be ~E5.5–7.25).[24,25] Indeed, the average patch size of contiguous marked cells in longitudinal sections of the adult distal ascending aorta is only 1.2 ± 0.1 cells (Supplementary Fig. 2). Therefore, at ~E5.5–7.25, there are multiple aortic wall progenitors, and the progeny of these progenitors mix extensively during embryogenesis, and this mixing persists. These findings are independently validated by our studies with female mice hemizygous for an X-chromosome-linked GFP transgene.[26] (Fig. 1i, j). In the early female mammalian embryo, one of two X-chromosomes in each somatic cell is randomly inactivated, and this process is complete by ~E7 in the mouse.[26] We observed a mixture of GFP+ and GFP− cells in the embryonic and postnatal aorta suggesting that the developing aortic wall derives from multiple polyclonal cells present at E7 or thereafter. Finally, clonal analysis of initial inner layer SMC marker− cells in the developing aorta indicates that their progeny migrate radially, longitudinally, and circumferentially within the media (Fig. 1k, l, Supplementary Fig. 3 and Supplementary Table 2; Methods).

Descending aortic SMCs derive from the presomitic mesoderm.[27,28] and ROSA26RCreER(Rb) embryos induced by threshold doses of 4-OH-T at E5.25 often contained descending aortic SMCs marked with one color, as well as cells in an adjacent mesoderm-derived tissue (i.e., lung mesenchyme; Fig. 1d, g) marked with a single distinct color.
Thus, at ~E5.5–7.25, the mesoderm and endoderm are differentially specified as are progenitors of the aortic wall and lung mesenchyme (derived from lateral plate mesoderm; Fig. 1h). Similar to the aorta, substantial migration and mixing of the progeny of early lung progenitors are indicated by the diffuse pattern of marked cells in the mesenchyme and epithelium.

**Most atherosclerotic plaque cells derive from one progenitor.**

We next evaluated whether the extensive SMC progenitor migration and mixing, which occurs during aortic morphogenesis also characterizes the accumulation of excess SMC-derived cells during atherosclerosis. *ApoE^−/−*, ROSA26R(Rb+)/ mice also carrying SMMHC-CreER^{12} or SMA-CreER^{12} were induced with tamoxifen (1 mg/day for 5 days), rested for 5 days, and fed a high fat diet (HFD) for 6, 12, or 16 weeks. Eleven aortic root atherosclerotic plaques were analyzed, and in ten plaques, all labeled cells were a single Rb color (Fig. 2a–c, Supplementary Fig. 4a and Supplementary Table 3), and in one plaque (at 16 weeks), 608 of the 613 labeled cells were Cerulean^+ and the remaining 5 cells were mCherry^+ (Supplementary Table 3). In terms of smooth muscle-derived cells, brachiocephalic plaques are similarly monoclonal (Supplementary Fig. 4b). With increasing HFD duration, labeled cells constituted a higher percentage of total aortic plaque cells (remarkably, 57 ± 7% by 16 weeks; Fig. 2d and Supplementary Table 3). Thus, a single pre-existing SMC is the source of the majority of cells of an advanced atherosclerotic plaque, and the smooth muscle progenitors undergo robust clonal expansion during plaque formation.
In addition to SMCs, macrophages play key roles in atherogenesis, but the clonal architecture of macrophages in plaques has not been studied. Mice carrying the inducible Csf1r-Mer-iCre-Mer mice were used to label monocytes or macrophages (Supplementary Fig. 5a). ApoE<sup>−/−</sup> Csf1r-Mer-iCre-Mer, ROSA26R<sup>(Rb+/+)</sup> mice were induced with tamoxifen (1 mg/day for 20 days), rested, and then fed a HFD for 6 weeks. At this time point, GFP<sup>+</sup> cap cells in human and murine plaques are hyperproliferative. Given the marked clonal expansion of a single smooth muscle-derived cell in atherogenesis, we evaluated cellular proliferation in the plaque. Our studies with human samples and high fat fed ApoE<sup>−/−</sup> mice indicate that cap cells are ~5 times more proliferative than cells in the core (Fig. 4a, b, d). In addition, the large majority of proliferating cells are SMC-derived, both in the cap (94%–99%) and in the core (70%–78%) (Fig. 4c). Proliferation of GFP<sup>+</sup> cap cells is independent of SMA expression as there is no difference in the percentage of pH3<sup>+</sup> cells in SMC<sup>+</sup> GFP<sup>+</sup> or SMC<sup>−</sup> GFP<sup>+</sup> cells (Supplementary Fig. 7). Taken together, these data suggest that a single pre-existing SMC gives rise to highly proliferative SMC marker<sup>+</sup> cells that initially coat the cap. In turn, these cap SMCs or their progeny migrate into the core and downregulate SMC markers (Fig. 4f, schematic).

Reduced integrin β3 levels induces SMC transdifferentiation. We next sought molecular regulators that modulate SMC fate and recruitment to the atherosclerotic plaque. Integrins are heterodimeric receptors that link the extracellular matrix to the cytoskeleton, and deletion of the gene encoding integrin β3 (Igfb3) exacerbates atherosclerosis burden in atheropane mice<sup>16</sup>. In high fat-fed ApoE<sup>−/−</sup> mice, β3 is highly expressed in SMC<sup>+</sup> cells of the tunica media and cap (that are derived from pre-existing SMMHC<sup>+</sup> cells) but not in SMC<sup>−</sup> core cells (Fig. 5a and Supplementary Fig. 8), which mimics the pattern of β3 expression in human atherosclerosis<sup>30</sup>. In contrast, in GFP<sup>+</sup> (SMC-derived) SMMHC<sup>+</sup> and SMC<sup>−</sup> cap cells continue to express PDGFR-β (Fig. 3b–d and Supplementary Fig. 6).

Timeline of the SMC lineage during atherogenesis. We next evaluated the temporospatial pattern of the contribution of SMC-derived cells to the atherosclerotic plaque in mice maintained on a HFD for increasing durations. After 5 weeks of high fat feeding, the plaques of ApoE<sup>−/−</sup> mice contain many cells expressing CD68 (Fig. 3a). Based on fate mapping with ApoE<sup>−/−</sup>, SMMHC-CreERT<sup>2</sup>, ROSA26R<sup>(mTmG/+)</sup> mice induced with tamoxifen and then maintained on a HFD, these early CD68<sup>+</sup> cells do not derive from pre-existing SMCs (i.e., GFP<sup>−</sup>; Fig. 3b). SMC-derived cells (i.e., GFP<sup>+</sup>) expressing SMA initially enter the plaque at the shoulder of the cap at 5.5 weeks (Fig. 3b). By 6 weeks, GFP<sup>+</sup>SMA<sup>+</sup>SMMHC<sup>+</sup> cells cover most of the cap, and SMA<sup>+</sup> cap cells express the undifferentiated mesenchyme marker PDGFR-β (Fig. 3b–d and Supplementary Fig. 6). At 8 weeks, SMC-derived cells are present in the internal region (or core) of the plaque and many have downregulated SMC markers. This process continues and by 12 weeks of high fat feeding, a substantial portion of core cells are GFP<sup>+</sup> and have low or no SMC marker expression (Fig. 3b). At this time point, GFP<sup>+</sup> cells that cover the cap remain SMC<sup>−</sup>SMMHC<sup>+</sup>, and SMC<sup>−</sup> cap cells continue to express PDGFR-β (Fig. 3b–d and Supplementary Fig. 6).

Cap cells in human and murine plaques are hyperproliferative. Given the marked clonal expansion of a single smooth muscle-derived cell in atherogenesis, we evaluated cellular proliferation in the plaque. Our studies with human samples and high fat fed ApoE<sup>−/−</sup> mice indicate that cap cells are ~5 times more proliferative than cells in the core (Fig. 4a, b, d). In addition, the large majority of proliferating cells are SMC-derived, both in the cap (94%–99%) and in the core (70%–78%) (Fig. 4c). Proliferation of GFP<sup>+</sup> cap cells is independent of SMA expression as there is no difference in the percentage of pH3<sup>+</sup> cells in SMC<sup>+</sup> GFP<sup>+</sup> or SMC<sup>−</sup> GFP<sup>+</sup> cells (Supplementary Fig. 7). Taken together, these data suggest that a single pre-existing SMC gives rise to highly proliferative SMC marker<sup>+</sup> cells that initially coat the cap. In turn, these cap SMCs or their progeny migrate into the core and downregulate SMC markers (Fig. 4f, schematic).
cells of the advanced atherosclerotic plaque of ApoE\(^{-/-}\), SMMHC-CreERT\(^2\), ROSA26R\((mTmG^+/-)\) mice, the scavenger receptor CD36 is primarily expressed in the region of the core that is adjacent to the cap and not in GFP\(^+\) cells of the cap itself (Fig. 5b). In the background of high fat fed ApoE\(^{-/-}\) mice, Itgb3 deletion results in expression of the macrophage marker CD68 in many SMMHC\(^+\) cells in the plaque and adjacent media of the atherosclerotic aorta (Fig. 5c and Supplementary Fig. 9).

Complementing these in vivo studies, reduction of Itgb3 in SMCs isolated from atheroprone mice increases Cd36 levels (Fig. 5d)\(^16\) but does not alter expression of other major cholesterol receptors/transporters Ldlr, Lrp1, Abca1 (Supplementary Fig. 10a). As a result, we observed an enhanced SMC uptake of ox-LDL (Fig. 5e). Itgb3 silencing does not alter Myh11 levels in isolated ApoE\(^{-/-}\) SMCs under basal conditions (Supplementary Fig. 10b); however, with cholesterol loading, in comparison to pre-treatment with scrambled siRNA, Itgb3 siRNA pre-treatment results in reduced levels of SMC differentiation markers Myh11 and Smtn, and increased levels of macrophage markers Csf1r, Cdi1b, Lgals3, and Lamp2 (Fig. 5f).

We next investigated further molecular mediators that may contribute to the effects of Itgb3 deficiency on the conversion of SMCs to macrophage-like cells. The pattern recognition receptor TLR4 and the pluripotency factor KLF4 have each been implicated in phenotypic changes of SMCs\(^3\), 31. siRNA-mediated knockdown of Itgb3 in aortic SMCs isolated from ApoE\(^{-/-}\) mice enhances Tlr4 levels (Fig. 5d) without altering levels of Klf4 (Supplementary Fig. 10b). Furthermore, on the background of Itgb3 silencing in ApoE\(^{-/-}\) SMCs, Tlr4 knockdown attenuates Cd36 levels (Fig. 5g) and with cholesterol loading, induces expression of SMC markers while attenuating macrophage marker levels (Fig. 5h). Thus, reduction of Itgb3 in SMCs increases Tlr4 levels, which facilitates Cd36 expression and cholesterol-induced transdifferentiation to a macrophage-like phenotype (Fig. 5i, schematic).

β3 in bone marrow-derived cells regulates SMC clonality. In addition to SMC fate, we found that in contrast to the monclonal plaques in Itgb3 wild-type mice (see Fig. 2a–c and Supplementary Fig. 4), Itgb3\(^{-/-}\), ApoE\(^{-/-}\), SMMHC-CreERT\(^2\), ROSA26R\((mTmG^+/-)\) mice fed a HFD have polyclonal SMC-derived cells in the atherosclerotic plaque as evidenced by multi-color labeling of plaque cells (Fig. 6a, b, Supplementary Fig. 11 and Supplementary Table 4). The multiple patches of cells...
of distinct colors with minimal mixing suggest that multiple SMC progenitors enter the plaque and then clonally expand within the plaque with limited migration. At 6 weeks of high fat feeding, labeled cells constitute a ~7-fold higher percentage of total plaque cells in \( \text{Itgb}3^{-/-} \) null mice, compared to that of wild-type \( \text{Itgb}3 \) mice (56 ± 11% vs. 8 ± 3%; Fig. 6c).

Based on these results with global \( \text{Itgb}3 \) null mice, as well as a previous study demonstrating that transplant of \( \text{Itgb}3^{-/-} \) bone marrow into \( \text{Itgb}3^{+/+} \) atheroprone mice worsens atherosclerosis in high fat fed mice\(^{17}\), we postulated that \( \text{Itgb}3^{-/-} \) bone marrow-derived cells induce multiple SMCs to migrate into the plaque. To test this hypothesis, bone marrow was harvested from experimental \( \text{Itgb}3^{-/-} \), \( \text{ApoE}^{-/-} \) or control \( \text{Itgb}3^{+/+} \), \( \text{ApoE}^{-/-} \) donor mice, and transplanted into \( \text{Itgb}3^{+/+} \), \( \text{ApoE}^{-/-} \), \( \text{SMMHC-CreER}\)^\(^{25}\), \( \text{ROSA}26\text{R}^{(Rho^{-/-})} \) recipient mice. There are similar numbers of total leukocytes, neutrophils, lymphocytes, macrophages, red blood cells, and platelets in the peripheral blood of experimental and control donors (Supplementary Fig. 12). Recipient mice were induced with tamoxifen, rested, irradiated, and then transplanted with experimental or control bone marrow. Four weeks after transplant, recipients were initiated on a HFD for 6–12 weeks. Similar to untransplanted...
**Fig. 4** Cap cells and smooth muscle-derived cells in the plaque are highly proliferative. **a–c** ApoE(-/-), SMMHC-CreERT2, ROSA26R(Rb1CreERT2) mice were induced with tamoxifen and fed a HFD for 6, 12, or 16 weeks. In **a** a transverse aortic root sections were stained for phosphohistoneH3 (pH3; mitotic marker), GFP (fate marker), SMA, and nuclei (DAPI). In **b** percentage of total cap or core cells that are pH3+ is shown: *vs. 6, 12 weeks, p < 0.05. In **c** percentage of pH3+ cells in the cap or core that are GFP+ is displayed. Percentages in **b, c** are quantified from images as shown in **a**, n = 3 mice for each time point and 10 sections per mouse. One-way ANOVA with Tukey’s multiple comparisons test and Student’s t-test were used. Per convention, the cap is defined as plaque region within 30 μm of lumen boundary for mice maintained on HFD for 12 or 16 weeks and within 15 μm for 6 weeks of HFD (see Methods). Cap cells and smooth muscle-derived plaque cells are highly proliferative. **d** Section of atherosclerotic left main coronary artery from a 64-year-old man stained for proliferation marker Ki67 (arrowheads), SMA, and nuclei (DAPI). Region in green box is shown below as close-ups. **e** Average of total Ki67+ cells in core or cap per human subject relative to area of the compartment and normalized to this quotient for the core. Quantification was conducted on images as shown in **d**. A total of 84 Ki67+ cells from atherosclerotic artery samples of six humans were scored for location. Student’s t-test was used. **f** Schematic depicts the accumulation of SMC-derived cells in atherosclerotic plaque. At 5.5 weeks of HFD in ApoE(-/-) mice, a single SMC enters the cap at the shoulder. The lineage of this progenitor SMC is highly proliferative and rapidly covers the cap by 6 weeks. By 8 weeks, these SMC-derived cells begin to migrate into the core and by 12 weeks, most SMC-derived cells in the core have downregulated SMC markers. Error bars represent standard deviations. Lu, lumen; Med, tunica media; Pl, plaque. Scale bars, 25 μm (**a**), 100 μm (**d**).

Igfb3 wild-type mice (see **Fig. 2a–c** and Supplementary **Fig. 4**), plaques of recipient mice transplanted with control bone marrow contain a single Rb color, indicating that a single pre-existing SMC is recruited into the plaque (**Fig. 6d, f**). In contrast, recipient mice transplanted with experimental bone marrows have multi-color plaques, indicating polyclonality of SMC-derived cells (**Fig. 6e, f**).

These findings suggest that bone marrow-derived cells and most likely macrophages regulate the recruitment of SMC progenitors into the developing atherosclerotic plaque. To evaluate this hypothesis further, monocytes from the femurs of experimental Igfb3(-/-), ApoE(-/-) or control Igfb3(+/-), ApoE(-/-) mice were harvested and differentiated into macrophages in culture. Isolated ApoE(-/-) SMCs were then cultured in the presence of medium conditioned by these experimental or control macrophages and subjected to migration and proliferation assays. During the first 8 h of culture, experimental macrophage conditioned medium induces ApoE(-/-) SMC migration (**Fig. 6g, h**) without altering proliferation (Supplementary **Fig. 13**). Subsequently, ApoE(-/-) SMCs cultured with experimental conditioned medium for two days are more proliferative than those cultured in control medium (**Fig. 6i, j**). In sum, β3 is required in bone marrow-derived cells (and probably in macrophages specifically) for limiting the number of source SMCs recruited into an atherosclerotic plaque to a single cell (**Fig. 6k**, schematic) as Igfb3(-/-) macrophages enhance SMC migration and proliferation.

**Discussion**

Herein, we use clonal analysis to compare and contrast the biology of SMC progenitors in the morphogenesis of the normal aortic wall and the pathogenesis of atherosclerosis. Based on our multiple independent single cell resolution clonal analyses in the mouse, we conclude that normal embryonic and adult aortic wall cells derive from multiple progenitors that are present at the time of gastrulation and whose progeny undergo robust mixing and migration (**Fig. 1**). Classical X-chromosome-linked inactivation studies of micro-dissected aortic segments from adult women heterozygous for genes on the X-chromosome, initially used zymography and subsequently PCR analysis as a crude clone marker. PCR results demonstrated that ~25% of samples from the normal aortic media were skewed to the same allele and were interpreted as suggesting that the aorta forms by expanding coherent smooth muscle clones with minimal mixing of adjacent clones. The difference between the conclusions in regard to normal aortic media morphogenesis of these human studies and our high-resolution investigations in mice likely reflect inherent limitations of the human studies and/or differences between species.

In addition to vessel development, we studied the clonal architecture of cells derived from monocytes/macrophages or SMCs in atherosclerotic plaques. Although macrophages largely accumulate in plaques through local proliferation, our results suggest that multiple macrophages are recruited into the plaque (Supplementary **Fig. 5**). In contrast, our studies utilizing ApoE(-/-), SMMHC-CreERT2, ROSA26R(Rb1CreERT2) mice fed a HFD for 6, 12, or 16 weeks demonstrate that in 91% (10 out of 11) of the atherosclerotic plaques analyzed, a single SMC gives rise to all smooth muscle-derived cells which comprise the majority of the total cells in an advanced plaque (**Fig. 2**). The one oligoclonal plaque observed was a 16-week plaque with two SMC progenitors, one of which dominated giving rise to more than 99% of SMC-derived cells (Supplementary **Table 3**).

These results must be considered in light of other investigations as the clonality of SMC-derived cells in atherosclerotic plaques is controversial. For instance, the authors of a study of tritiated thymidine labeling patterns in high cholesterol fed swine interpreted their results as indicating polyclonal plaques. In contrast, a study analyzing X-chromosome inactivation in adult women indicated that the normal human aortic wall was subsequently suggested to be composed of large monoclonal SMC patches; however, because the normal human aortic wall was subsequently suggested to be composed of large patches of SMCs with the same X-chromosome inactivation pattern (see above), such approaches cannot distinguish whether a single SMC or multiple SMCs located in proximity in the normal wall give rise to plaque cells. In addition, low-resolution X-gal images of fate-mapped cells expressed the SMC marker SM22α in 1-year-old normal mouse (cholesterol-fed ApoE(-/-)) mice suggest that clonal expansion of a single pre-existing SM22α+ cell may contribute to regions of plaques.

Most recently, two studies of SMMHC-CreERT2 mice also carrying the multi-color ROSA26R(Confetti) Cre reporter in atheroprone models have analyzed the clonal architecture of SMC-derived plaque cells. In the first study, these mice were on an ApoE(-/-) background, induced with tamoxifen (10 mg) over two weeks and then fed a HFD for 16–19 weeks. One-half of the plaques contained SMC-derived cells of a single color (monoclonal) and the other half contained SMC-derived cells of two or, in 8% of cases, more colors (oligoclonal or polyclonal). In the second study, the mice were induced with tamoxifen (1 mg/day for 10 days) and then infected with rAAV8-mD377Y-mPCSK9 virus particles and maintained on a HFD for 12, 24, or 36 weeks. The authors concluded that all SMC-derived plaque cells result "from a very limited number of medial SMCs undergoing coherent oligoclonal proliferation." The different results between these recent studies and our study are likely due to the atherosclerosis model, the HFD duration in ApoE(-/-)
mice as all plaques that we analyzed at 6 or 12 weeks \((n = 7)\) were monoclonal and/or a difference between the Confetti and Rb reporters. Indeed, the Confetti reporter alters PCSK9/HFD-induced atherosclerosis burden\(^\text{15}\). This Cre reporter includes multiple loxP sites that are oriented in opposite directions, and thus, Cre will invert the intervening sequence resulting in the expression of a different fluorophore\(^\text{33, 34}\). Hence, if proliferation occurs in a labeled parental SMC progenitor during the tamoxifen phase, the parental and daughter cells may have distinct colors. In contrast, the Rb reporter utilized in our studies contains pairs of incompatible loxP sites in the same orientation such that only a single mutually exclusive recombination event can occur, resulting in the indelible expression of a single color\(^\text{22, 35, 36}\).

Thus, taking together the results of our study and those of other studies also using modern genetic marking approaches, multiple SMC progenitors mix and migrate widely in the morphogenesis of the normal aortic wall, whereas a single (or at most, rare) SMC marker\(^+\) cell(s) in the adult ApoE\(^{-/-}\) aorta are recruited into the early atherosclerotic plaque and clonally expands. However, the SMC marker\(^+\) progenitor cell or pool of cells that is recruited into the early atherosclerotic plaque has not been identified in the aortic media. We previously identified in...
the normal lung, a pool of specialized SMC progenitors located at the muscular–unmuscular arteriole border, and with hypoxia exposure, one of these progenitors migrates distally and clonally expands giving rise to pathological distal arteriole SMCs. Further studies are needed to evaluate whether there is a similar specialized pool of rare SMC marker + progenitors in the normal aortic wall that are capable of being recruited into and clonally expanding in early adjacent plaques upon high fat feeding. An alternative hypothesis is that a progenitor SMC is stochastically selected among a level playing field of SMMHC + cells to enter the plaque and proliferate, and this cell and/or its progeny exert an inhibitory influence on other medial SMCs preventing them from...
entering the plaque. If a pool of SMC progenitors that expand in atherosclerosis is identified, therapies targeting such cells have far-reaching implications.

Similar to our prior studies of hypoxia-induced pulmonary arteriolar remodeling \(^{26, 37}\), herein, a time course of smooth muscle-derived cells during HFD-induced atherosclerosis reveals critical insights into pathogenesis (Fig. 3). The cells of the early atherosclerotic plaque are CD68\(^{+}\) and do not derive from SMCs but instead likely come from monocyte recruitment and/or perhaps local macrophage proliferation in the plaque\(^{32}\). Subsequently, the lineage of a single SMC covers the cap. These cap cells have a unique molecular signature expressing markers of SMCs (SMAMHC, SMMHC) and undifferentiated mesenchyme (PDGFR-\(\beta\)) (Fig. 3 and Supplementary Fig. 6), and our studies of both human and murine plaques indicate that the cap cells are 5-fold more proliferative than core cells (Fig. 4). After giving rise to SMC marker\(^{+}\) cap cells, smooth muscle-derived cells populate the core of the plaque, and core cells have low expression of SMC markers. These results suggest that the smooth muscle-derived SMC marker\(^{+}\) cap cells are the source of many of the SMC marker\(^{-}\) cells in the internal region of the plaque. In previous fate mapping studies of 18-week high fat fed ApoE\(^{−/−}\) mice, Shankman et al. reported that 82% of SMC-derived cells assume SMA\(^−\) fates, including LGALS3\(^{+}\) macrophage-like cells, in atherosclerotic plaques\(^{5}\). Furthermore, deletion of Klf4 in SMCs attenuated their transdifferentiation to LGALS3\(^{+}\) plaque cells and reduced plaque size, suggesting that SMC-derived SMA\(^−\) cells have deleterious effects in atherosclerosis\(^{5}\). Cap SMCs are widely implicated in stabilizing plaques against rupture, but our results suggest that these cells also have a negative impact on plaque dynamics by giving rise to pathological SMC marker\(^{−}\) core cells. Strategies to target SMC marker\(^{+}\) cap cells and prevent them from transdifferentiating and migrating into the core are needed.

In an atheroprone background (ApoE\(^{−/−}\) or Ldlr\(^{−/−}\)), deletion of Itgb3 exacerbates HFD-induced atherosclerosis\(^{36, 15}\), but underlying mechanisms are not well understood. Herein, we demonstrate that in SMC-derived cells, integrin \(\beta 3\) is expressed on SMC marker\(^{+}\) cap cells but not on core cells whereas CD36 is primarily expressed in core cells (Fig. 5 and Supplementary Fig. 8). Integrin \(\beta 3\) has been implicated in regulating SMC migration and proliferation\(^{16−20}\), but not marker expression. The aortic media and plaque of high-fat fed ApoE\(^{−/−}\), Itgb3\(^{−/−}\) mice harbor many cells expressing markers of both SMCs (SMMHC and macrophages (CD68)), and knockout of Itgb3 in ApoE\(^{−/−}\) SMCs induces scavenger receptor Cd36 expression and ox-LDL uptake and facilitates cholesterol-induced transdifferentiation to a macrophage-like phenotype (Fig. 5). TLRs are the best characterized class of pattern-recognition receptors of the innate immune system, and recently, ox-LDL treatment of human arterial SMCs have been shown to induce TLR4 expression\(^{40}\). Herein, we demonstrate that knockout of Itgb3 enhances TLR4 levels in SMCs, and in the context of Itgb3 knockout, TLR4 reduction attenuates both Cd36 expression and cholesterol-induced transdifferentiation to a macrophage-like phenotype. Taken together, we speculate that in the early plaque, down-regulation of integrin \(\beta 3\) in SMC\(^{+}\) cells or their progeny facilitates migration into the core, TLR4-induced CD36 expression, cholesterol uptake, and transdifferentiation.

No molecular or cellular players have previously been identified that regulate clonality of smooth muscle-derived cells in atherosclerosis. Our studies demonstrate that in contrast to Itgb3\(^{−/−}\), ApoE\(^{−/−}\) mice, multiple pre-existing SMCs are recruited into the plaques of Itgb3\(^{+/−}\), ApoE\(^{−/−}\) mice (Fig. 6). Transplant of Itgb3\(^{+/−}\), Ldlr\(^{−/−}\) bone marrow into Itgb3\(^{+/+}\), Ldlr\(^{−/−}\) mice worsens HFD-induced atherosclerosis\(^{17}\), and remarkably, we show that transplant of Itgb3\(^{−/−}\), ApoE\(^{−/−}\) bone marrow into Itgb3\(^{+/+}\), ApoE\(^{−/−}\) mice results in polyclonal smooth muscle-derived plaque cells. These results indicate that Itgb3 null bone marrow-derived cells (most likely macrophages) are integral in the recruitment of multiple SMCs into the atherosclerotic plaque. There is a dearth of investigations into the potentially critical effects macrophages and macrophage-derived factors play in modulating the biology of SMCs in general and especially in the context of atherosclerosis. Our findings with isolated cells indicate that medium conditioned by Itgb3\(^{−/−}\) macrophages induces substantially more migration and proliferation of aortic SMCs compared to medium conditioned by macrophages wild type for Itgb3. Interestingly, integrin \(\beta 3\) is not detectable in macrophages of early plaques by immunohistochemistry (Supplementary Fig. 8). Thus, we speculate that Itgb3\(^{−/−}\) monocytes are altered such that when they enter the plaque and become macrophages, their gene expression differs from wild-type macrophages, and this difference includes secreted factors that directly induce multiple SMCs to migrate into the lesion and proliferate. An alternative or additional plausible explanation for the in vivo findings is that Itgb3\(^{−/−}\) bone marrow-derived cells signal to non-smooth muscle cells in the blood vessel/plaque milieu, such as endothelial cells, that in turn activate SMCs.

In sum, the increased number of recruited SMC progenitors and altered fate of these cells in Itgb3 mutant mice are likely major factors exacerbating atherosclerosis. More broadly, we speculate that the number of smooth muscle-derived progenitors recruited into a plaque is a key determinant of the rate of progression of atherosclerosis. Thus, therapeutic approaches that reduce the recruitment and alter the fate of such progenitors into human atherosclerotic plaques warrant intense investigation.
Methods

Animals. Mouse experimental protocols were approved by the IACUC at Yale University, and we complied with all relevant ethical regulations. Wild type (C57BL/6, FVB), ApoE<sup>−/−</sup>, ROSA26<sup>CreERT2</sup>, ROSA26<sup>−/−mTmG<sup>lox/lox</sup></sup>, Igκ<sup>−/−</sup>, and X-linked D4I/XGFP (denoted herein as XGFP<sup>−/−</sup>) mice were purchased from Jackson Laboratories<sup>8,28,41,42</sup>. Mice carrying an inducible Cre recombinase, SMHHCCreERT2<sup>−/−</sup>, SmcCreERT2<sup>−/−</sup>, Csf1r-Mer-Cre-Mer<sup>−/−</sup>, or the multi-color Rainbow (Rb) Cre reporter<sup>43–45,39,44,44</sup> have described in Supplementary Table 1 with both: (i) single labeled cells in one of the tissues (i.e., aortic wall or lung mesenchyme) and (ii) in the other tissue, either no marked cells or only 17 embryos had labeled cells of the same color in the aortic wall and lung mesenchyme. In addition, for longitudinal aortic sections, dams pregnant with ROSA26<sup>CreERT2</sup> embryos were induced with a single limiting 4-OH-T dose (20 µg at E5.25, and then embryos were allowed to develop and were analyzed during adulthood (8 weeks). Serial 10-µm-thick longitudinal sections of the distal thoracic descending aorta (320 µm) were stained and imaged as described for transverse sections above. Of the embryos induced (n = 10), 60% had no marked cells and 40% were labeled with a single color (Supplementary Table 1). Per each marked cell or group of contiguous marked cells, we scored the number of contiguous marked cells.

Dams pregnant with SMHHCCreERT2<sup>−/−</sup> embryos that were also heterozygous for a Cre reporter (i.e., ROSA26<sup>−/−mTmG<sup>lox/lox</sup></sup>) or ROSA26<sup>−/−mR<sup>lox/lox</sup></sup>) were induced with a single limiting tamoxifen (0.5 mg) injection at E9.5. Embryos were allowed to develop until E14.5 or E16.5 when the distal descending aorta was sectioned (60 consecutive sections of 10 µm thickness), stained (with DAPI and in the case of GFP clones, also for GFP, SMA, and CD31), and analyzed. Each marked cell was scored based on position and specific layer in the aorta and, in the case of the Rb Cre reporter, on color (i.e., Cerulean, mOrange, or mCherry). Of all embryos analyzed (n = 29), 48% had aortas containing marked cells (n = 14, see Supplementary Table 2) while 52% of aortas were not labeled. Of the SMHHCCreERT2<sup>−/−</sup>, ROSA26<sup>−/−mR<sup>lox/lox</sup></sup> embryos analyzed with marked aortic SMCs (n = 7), all of them were of a single color. Higher tamoxifen doses (0.6–2 mg) resulted in more embryos with marked cells (74%), frequent multi-color labeling (67%) and more cells marked. In contrast, lower doses (0.125–0.4 mg) did not induce any marked aortic cells (n = 23).

Immunohistochemistry. Following euthanasia, embryos, pups, or dissected adult aortas were fixed in 4% paraformaldehyde for 2 h at 4 °C. Tissue was then incubated in 30% sucrose solution in optical tissue cryomedium (Tissue Tek), and stored at −80 °C. Tissue was cryosectioned (10 µm) in the transverse axis, and sections were incubated with blocking solution (5% goat serum in 0.1% Triton X-100 in PBS (PBS-T)) and then with primary antibodies diluted in blocking solution overnight at 4 °C. On the next day, sections were washed with PBS-T and then incubated with secondary antibodies for 1 h. Primary antibodies used were anti-CD31 (1:500, BD Pharmingen, 553370), anti-SMMHC (1:100, Novus Biologicals, NB400–647), anti-GFP (1:500, Invitrogen, A11122; 1:100, R&D, AF1042). For amplification of the PDGFR-β signal, sections were incubated with the biotin-conjugated antibody and then with the ABC Elite reagent (vector), and DAB as the substrate (Dako). Sections were counterstained with DAPI. Secondary antibodies were conjugated to either FITC or Alexa-488, and labeled nuclei were visualized using fluorescence microscopy. Sections were counterstained with DAPI and in the case of the Rainbow reporter, on color (i.e., Cerulean, mOrange, or mCherry). Of all embryos analyzed, 60% had no marked cells, 20% had single-color marked cells, and 20% had multi-color labeled cells of contiguous marked cells. In contrast, lower doses (0.125–0.4 mg) did not induce any marked aortic cells (n = 23).

Immunohistochemistry. Following euthanasia, embryos, pups, or dissected adult aortas were fixed in 4% paraformaldehyde for 2 h at 4 °C. Tissue was then incubated in 30% sucrose solution in optical tissue cryomedium (Tissue Tek), and stored at −80 °C. Tissue was cryosectioned (10 µm) in the transverse axis, and sections were incubated with blocking solution (5% goat serum in 0.1% Triton X-100 in PBS (PBS-T)) and then with primary antibodies diluted in blocking solution overnight at 4 °C. On the next day, sections were washed with PBS-T and then incubated with secondary antibodies for 1 h. Primary antibodies used were anti-CD31 (1:500, BD Pharmingen, 553370), anti-SMMHC (1:100, Biomedical Technologies, J64817), anti-GFP (1:500, Invitrogen, A11122; 1:100, Abcam, ab13970), anti-pher (1:200, Millipore, 06–570), anti-Ki67 (1:100, Vector Labs, VP-RM04), anti-integrin β3 (1:200, Abcam, ab57872 and ab197662), anti-CD31 (1:200, Biolegend, MA1957), anti-D3E4 (1:100, Novus Biologicals, NB400–144), directly conjugated Cy3 anti-βMA (1:500, Sigma-Alrich, C6198), anti-PDGF-β (1:100, Abcam, ab32570), and biotinylated anti-PDGF-β (1:50, R&D, AF1042). For amplification of the PDGFR-β signal, sections were incubated with the biotin-conjugated antibody and then with the ABC Elite reagent (vector), and DAB as the substrate (Dako). Sections were counterstained with DAPI. Secondary antibodies were conjugated to either FITC or Alexa-488, –564, –654 fluorophores (1:1000, Invitrogen). Nuclei were visualized by DAPI staining (1:1000, Sigma-Alrich, D9542). Note, membrane-localized tdTomato (mt) fluorescence in the aorta of ROSA26<sup>−/−mTmG<sup>lox/lox</sup></sup> mice is very weak and essentially undetectable in comparison to the strong fluorescence in the red channel of staining regimens with antibodies directed against SMA or SMHC.

Left main coronary artery atherosclerosis specimens from adult patients (35 ± 11 years old; 2/3 men, 1/3 women) were fixed in formalin, paraffin-embedded, and sectioned<sup>25</sup>. Sections of human arteries were treated with xylene to permit paraffin embedding and then dehydrated into water. Rehydrated sections were washed with TONT (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.2% Tween-20) and incubated for 20 min in boiling 10 mM sodium citrate, pH 6.0 for antigen retrieval. Sections were then immersed in cold water and immunostained as described above for cytoskeleton except washes were done in TONT. As previously described<sup>44</sup>, the ratio of the thickness of the intima regimens with antibodies directed against SMA or SMHC.

Aortic SMC isolation. Aortic SMCs were isolated by modifications of procedures described previously<sup>45</sup>. Briefly, aortas from the root to the iliac bifurcation were dissected from adult ApoE<sup>−/−</sup> mice and opened longitudinally, and endothelial cells and the adventitia were gently removed. The aortas were sequentially washed with 1% penicillin/streptomycin in PBS and then with 100% fetal bovine serum (FBS). Washed aortas were cut into small pieces and cultured in plastic dishes in DMEM supplemented with 20% FBS, 1% penicillin/streptomycin. After 3 days, the medium was replaced by fresh medium that was similar except with 10% of the media changed with 10% FBS. Aortic SMCs that migrated out of the aortic pieces and adhered to the dish were trypsinized, expanded, and passaged. For experiments, cells were used until the fifth passage.

siRNA-mediated knockdown of Igκb3. Isolated murine aortic SMCs were transfected with Lipofectamine RNAiMAX (Invitrogen) containing siRNAs (Origene) targeted against Igκb3 (30 nM) or scrambled RNA for 6 h. Cells were then washed in PBS and cultured in DMEM supplemented with 10% FBS for 72 h.

Loading with ox-LDL or water-soluble cholesterol. Following Igκb3 knockdown, SMCs were serum starved in DMEM with 0.2% BSA for 24–48 h and then loaded with LDL or cholesterol. For LDL experiments, SMCs were incubated with Dil-conjugated-LDL or ox-LDL (10 µg/ml, Alfa Aesar), 0.2% BSA in DMEM for 10 h at...
37 °C, washed with PBS, and then fixed with 3% paraformaldehyde. For water-soluble cholesterol experiments, SMCs were cultured with cholesterol: methyl-β-cyclodextrin complexes (10 μg/ml Sigma); 0.2% RSA in DMEM for 3 days, and then lysed for RNA isolation.

Quantitative real-time PCR analysis. RNA was isolated from aortic SMCs of ApoE−/− mice with the PureLink™ RNA Minikit (Invitrogen), and this RNA (0.2 μg) was reverse transcribed with the Script cDNA Synthesis Kit (Biorad). The expression levels of selected genes were determined by quantitative PCR and normalized to Gapdh. The following forward and reverse primer pairs were used for these genes (encoded protein): Abla1 (ABCA1) - GCTGTGGGAGATGGTTA-CCCTCCCTTTA C10338, C10338 donor mice by flushing with sterile Opti-MEM medium (Thermo Fisher). Each recipient mouse was injected retro-orbitally with 2 x 10^6 bone marrow cells. Four weeks after bone marrow transplantation, mice were switched to a HFD for up to 12 weeks and then euthanized. Transverse sections of the aortic root were stained, imaged, and quantified as noted above for clonal analysis.

Macrophage-conditioned medium. Bone marrow from the femurs of experimental Igkb3+/+, ApoE−/−, or control Igkb3−/−, ApoE−/− donor mice by flushing with sterile Opti-MEM medium (Thermo Fisher). Each recipient mouse was injected retro-orbitally with 2 x 10^6 bone marrow cells. Four weeks after bone marrow transplantation, mice were switched to a HFD for up to 12 weeks and then euthanized. Transverse sections of the aortic root were stained, imaged, and quantified as noted above for clonal analysis.

Proliferation and migration assays of cultured SMCs. For proliferation studies, ApoE−/− aortic SMCs were cultured in DMEM, 10% FBS, 1% penicillin-streptomycin until they reached ~60% confluency. Cells were then serum starved in DMEM overnight followed by incubation with macrophage-conditioned medium for 8 or 48 h. During the last 8 h of this incubation, EdU (10 μM) from the Click-IT EdU Alexa Fluor 555 Imaging Kit (Thermo Fisher Scientific, C10338) was added to the medium. Cells were then fixed in 4% PFA and permeabilized in 0.5% PBS-T; EdU staining was performed as per the manufacturer’s instructions.

For migration studies, cell culture inserts (Ibidi), composed of two chambers (2.4 cm in diameter, 25 μm in height) were used. Cells were serum starved overnight and washed prior to removal of the insert and cultured in macrophage-conditioned medium for 8 h. The cell coverage of the area that was blocked by the insert was measured immediately after insert removal and 8 h later.

Imaging. Images were acquired with Nikon microscopes (Eclipse 80i upright fluorescent or Eclipse TS100 inverted) or Leica S5P confocal microscope. For image processing, analysis and cell counting, Adobe Photoshop and Image J software were used.

Statistical analysis. Student’s t-test, one-way ANOVA with Tukey’s multiple comparisons test, and Chi-square test were used to analyze the data (Prism7 software). Statistical significance threshold was set at p ≤ 0.05. Tests assumed normal distribution and were two-sided, and all data are presented as mean ± standard deviation.

Data availability. All available data are available from the authors.

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Author Contribution

A.M., L.D., N.R., C.F.H., and D.M.G. conceived of and designed experiments, and A.M., Z.F., R.R.C., I.K., N.R., B.A., and L.D. performed them. A.M., L.D., Z.F., A.Q.S., and D.M. G. analyzed the results, and A.M., A.Q.S., and D.M.G. prepared the figures. G.T. and L.Q. contributed human samples. A.M. and D.M.G. wrote the manuscript.

Additional information

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