Smooth Muscle Cells in Atherosclerosis Originate From the Local Vessel Wall and Not Circulating Progenitor Cells in ApoE Knockout Mice

Jacob F. Bentzon, Charlotte Weile, Claus S. Sondergaard, Johnny Hindkjær, Moustapha Kassem, Erling Falk

Objective—Recent studies of bone marrow (BM)-transplanted apoE knockout (apoE−/−) mice have concluded that a substantial fraction of smooth muscle cells (SMCs) in atherosclerosis arise from circulating progenitor cells of hematopoietic origin. This pathway, however, remains controversial. In the present study, we reexamined the origin of plaque SMCs in apoE−/− mice by a series of BM transplantations and in a novel model of atherosclerosis induced in surgically transferred arterial segments.

Methods and Results—We analyzed plaques in lethally irradiated apoE−/− mice reconstituted with sex-mismatched BM cells from eGFPapoE−/− mice, which ubiquitously express enhanced green fluorescent protein (eGFP), but did not find a single SMC of donor BM origin among ~10 000 SMC profiles analyzed. We then transplanted arterial segments between eGFPapoE−/− and apoE−/− mice (isotransplantation except for the eGFP transgene) and induced atherosclerosis focally within the graft by a recently invented collar technique. No eGFP+ SMCs were found in plaques that developed in apoE−/− artery segments grafted into eGFPapoE−/− mice. Concordantly, 96% of SMCs were eGFP+ in plaques induced in eGFPapoE−/− artery segments grafted into apoE−/− mice.

Conclusions—These experiments show that SMCs in atherosclerotic plaques are exclusively derived from the local vessel wall in apoE−/− mice. (Arterioscler Thromb Vasc Biol. 2006;26:2696-2702.)

Key Words: atherosclerosis ■ smooth muscle cells ■ adult stem cells ■ pathology ■ apoE knockout mice

Recruitment of smooth muscle cells (SMCs) is a key mechanism in the development of atherosclerosis and its clinical manifestations. SMCs contribute to plaque volume through matrix synthesis, and the accretion of SMCs plays a decisive role in the pathogenesis of coronary stenosis. Conversely, paucity of SMCs in the fibrous cap of plaques increases the risk of plaque rupture and life-threatening arterial thrombosis. Understanding the origin of plaque SMCs may provide new opportunities for controlling their ambiguous nature.

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Until recently, the only source of SMCs in atherosclerotic lesions was considered to be the local vessel wall. According to this hypothesis, local SMCs in the arterial media and intima modulate into a synthetic and migratory phenotype (aka, phenotypic modulation) and form the fibrous component of the plaque. This theory was essentially inferred from a line of suggestive observations in arterial injury models in the 70s and 80s. More to the point, Cre/lox fate mapping in the apoE knockout (apoE−/−) mouse model of atherosclerosis recently confirmed that preexisting SMCs, presumably from the local media, contribute to plaque SMCs during atherosclerosis, but the existence of other sources could not be excluded by this technique.

In 2002, an alternative and major source of SMCs in atherosclerosis was reported, and this new paradigm has great impact on current research in this area. Based on observations in bone marrow (BM)-transplanted apoE−/− mice, it was concluded that a substantial fraction of plaque SMCs arise from circulating progenitor cells of hematopoietic origin. This pathway holds promise for the development of novel therapeutic means of controlling the recruitment and accumulation of SMCs in plaques. However, it remains questionable whether the quality of the histological documentation in that study can support a definitive conclusion, especially because the seeming use of unfixed tissue for detection of enhanced green fluorescent protein (eGFP) can lead to diffusion of the tracer marker from sectioned cells. Furthermore, reconstitution of apoE−/− mice with apoE−/−...
BM as performed in that experiment provides powerful protection against the development of atherosclerosis. To address these concerns, we repeated the BM transplantation experiment in apoE−/− mice with a number of methodological modifications but could not replicate the results. To confirm and extend these findings, we then studied atherosclerosis induced in surgically transferred arterial segments. Here, we show that atherosclerotic plaque SMCs are derived from the local vessel wall and not circulating smooth muscle progenitor cells in apoE−/− mice.

**Methods**

For a detailed Methods section, please see the online data supplement, available online at http://atvb.ahajournals.org.

**Transgenic Animals**

The Danish Animal Experiments Inspectorate approved all procedures. ApoE−/− mice (B6.129P2-Apoetm1Unc/J, Taconic M&B, Ry, Denmark), backcrossed more than 10 times to C57BL/6 mice, and eGFP+/− C57BL/6 mice (C57BL/6-Tg[ACTB-EGFP]1Osb/J; Jackson Laboratories, Bar Harbor, Me), were intercrossed to obtain eGFP+/+ C57BL/6 mice (apoE−/−, n=8) as tracers. *Per total number of nucleated SMaA profiles analyzed.*

**Bone Marrow Transplants**

ApoE−/− mice (n=28) were lethally irradiated and rescued with eGFP+/− C57BL/6 mice (apoE−/−) as tracers. One mouse died shortly after BM transplantation. Four randomly-chosen BM-transplanted mice were killed after 4 weeks. The other mice were killed shortly after BM transplantation. Four randomly-chosen BM-transplanted mice (apoE−/− CCA → apoE−/− transplanted mice, n=2) were treated identically with the exception that no collars were inserted.

**Immunohistochemistry**

SMCs were identified by staining for smooth muscle α-actin (SMaA). This abundant protein is considered the most sensitive, though not specific, SMC marker. SMaA is expressed early in embryonic development and lost late in phenotypic modulation, and it is the marker on which previous conclusions on BM origin of plaque SMCs have been based. Specificity of SMaA stainings was confirmed by observing the expected subplasmalemmal distribution of SMaA in plaque SMCs and by negative isotype control stainings. The Mac2 epitope was used as a marker for plaque macrophages.

**Fluorescence In Situ Hybridization**

The Y chromosome was visualized in a subset of SMaA stained aortic root sections. First, z-axis image stacks of SMaA− stained cell-containing areas were acquired and stored. Then, cover slips were removed and the immunostained sections were pretreated in 10 mmol/L sodium citrate, pH 6.0 (2 hours, 80°C) and 0.025% pepsin solution (10 minutes, 37°C, Sigma), which totally extinguished eGFP fluorescence and deteriorated the SMaA staining signal. Y chromosomes were then detected by a fluorescent isothiocyanate (FITC)-conjugated paint probe using the protocol recommended by the manufacturer (Cambio), and the phenotype of cells with Y+ nuclei was identified in the stored images. This sequential technique allows for a robust analysis, because the cell phenotype is analyzed before the harsh pretreatment necessary for fluorescence in situ hybridization (FISH) distorts morphology.

**Microscopic Analysis**

Sections were examined in an Olympus Cell-R epifluorescence microscope system equipped with differential interference contrast (DIC) optics and motorized focus. Deconvolution analysis on wide-field z-axis image stacks (0.3 μm optical thickness) was performed in a subset of sections (aortic root sections for sequential FISH analysis and CCA plaques) by using a blind 3D deconvolution algorithm (Autoquant Deblur 9.3; Autoquant Imaging). This tech-
niche, like confocal microscopy, yields high signal-to-noise images of thin optical sections.

SMαA+ cell profiles in the range of small SMC nuclei or larger (from ~3 μm [minor axis] × 5 μm [major axis]) were analyzed for colocalization. Because all SMCs contain a nucleus of relatively similar size, this strategy is an accurate analysis of the presence of eGFP+ SMαA+ cells and avoids many interpretational problems presented by smaller autofluorescent structures and overlapping parts of closely opposed eGFP+ and SMαA+ cells. The number of analyzed SMαA profiles was counted to estimate statistical power. In BM transplanted mice, we estimated the total number by counting a representative subset (34%) of the sections. Nucleated SMαA profiles, defined as cells where the nucleus was circumscribed by SMαA stain, were counted and analyzed for combined Y chromosome and SMαA signal.

Statistical Analysis

Rare binomially distributed events approach the Poisson distribution, and a 95% confidence limit for double-positive cells was calculated by this approximation (95% confidence limit: P<3.0/number of observations). Sections were taken at least 50 μm apart to ensure that the same SMC was not analyzed twice.

Results

Sex-Mismatched BM Transplantation

The degree of hematopoietic chimerism obtained in eGFP+apoE−/− BM → apoE−/− transplanted mice was assessed by flow cytometry of peripheral blood leukocytes. The fraction of fluorescent cells in BM transplanted mice 4 and 24 weeks after transplantation was 93.4 ± 2.8% (mean±SD, n=23) and 93.7 ± 1.4% (n=12), respectively, which was similar to that of eGFP+apoE−/− positive control mice (92.4±2.0% [n=4] and 92.2±0.8% [n=4], respectively; Figure 2a). The sustained presence of eGFP+ leukocytes documents the replacement of hematopoietic stem cells, which are the only long-term self-renewing cells in the hematopoietic system.17 Plasma lipid values are described in supplemental Table I.

Vascular Pathology in BM Chimeras

In animals euthanized 4 weeks after BM transplantation (n=4), only foam cell lesions were present at the arterial sites selected for analysis, excluding the possibility that SMCs had entered the intima already before full reconstitution of the hematopoietic system with eGFP+ cells was confirmed. At 20 weeks of age, all mice had developed fibrofatty plaques in the aortic root, and fibrofatty plaques were also present in the aortic arch, brachiocephalic trunk, abdominal aorta, and to a variable extent in the descending thoracic aorta at 32 weeks of age.

Atherosclerotic Plaque SMCs Are Not Derived From Hematopoietic Stem Cells

As an internal validation of the experiment, the hematopoietic origin of plaque macrophages was verified by the demonstration of cells double-positive for eGFP and the murine macrophage marker Mac2 in plaques in BM transplanted mice (Figure 2b). SMαA+ cells were predominantly located in the fibrous cap separating the core of the plaque from the arterial lumen. As expected, SMαA+ cells were almost uniformly eGFP+ in eGFP+apoE−/− positive control mice (n=4, 32 weeks of age, 580 of 598 SMαA+ cell profiles analyzed). In agreement with observations reported by others, SMαA expression was lost in major parts of the media underlying atherosclerotic plaques (Figure 3).7

We did not identify a single eGFP+SMαA+ cell among ~10 000 SMαA+ cell profiles analyzed in 154 sections from multiple sites of the arterial tree in 23 BM-transplanted mice (95% confidence limit <0.03%) (Figures 1a and Figure 3; supplemental Figure I). The identical result was reached using blind 3D deconvolution performed on a subset of the same sections from the aortic root (Figure 4).

As an independent tracing method, we detected Y chromosomes in SMαA immunostained sections from the aortic root (Figure 4). Not once did we convincingly detect a Y chromosome in 367 analyzed nucleated SMαA+ profiles in plaques from male-to-female BM-transplanted mice. In one inconclusive case, a SMαA+ profile circumscribed two nuclear profiles, one of which was Y chromosome+. A positive Y chromosome signal was detected in 114 of 209 analyzed nucleated SMαA+ cell profiles (54%) in female-to-male BM-transplanted mice, which was similar to that detected in

Figure 2. The hematopoietic system in irradiated apoE−/− mice was reconstituted with eGFP+apoE−/− cells after BM transplantation. a, Flow cytometry of peripheral blood leukocytes. Left panel, Cells within a defined forward-side scatter gate that encompassed the major leukocyte populations were analyzed. Right panel, Green fluorescence of leukocytes obtained from an apoE−/− mouse (top), an eGFP+apoE−/− mouse (middle), and an eGFP+apoE−/− BM → apoE−/− transplanted mouse (bottom). b, Macrophage foam cells detected by the murine macrophage marker Mac2 were eGFP+ in BM transplanted mice as expected. Aortic root plaque from eGFP+apoE−/− BM → apoE−/− transplanted mouse (32 weeks of age). Green indicates eGFP; Red, Mac2; Yellow, overlay of eGFP and Mac2; Blue, DAPI; Gray scale, DIC. Color channels are shown separately to facilitate interpretation. L indicates lumen; F, foam cells; M, tunica media. Scale bar=100 μm.
plaques from the control apoE−/− and eGFP−apoE−/− male mice (n=2+2, 32 weeks of age, 48 of 94 analyzed nucleated SMαA− cells) and in the range predicted from the thickness of the section and the nuclear size.

**SMCs Are Derived From the Local Vessel Wall in Cross-Grafted Arterial Segments**

Our observations in BM-transplanted mice showed that differentiation of hematopoietic stem cells to plaque SMCs is exceedingly rare if it occurs at all. To evaluate whether circulating smooth muscle progenitor cells of nonhematopoietic origin can contribute to atherosclerotic plaque SMCs, we then analyzed SMC origin in atherosclerotic lesions that were induced proximal to a constrictive collar in cross-grafted CCA segments (Figures 1b and 5a).

Of 20 mice in which we performed CCA segment transplantations and collar placements, complete or near-complete graft occlusions were present in 5 mice at time of sacrifice. In 4 mice, no significant lesions were found, and in 2 mice, extensive lesion formation extending into the graft from the proximal anastomosis was present. These were all excluded from the analysis. Most likely, the cause of these technical failures was inconsistency in tightening of the ligature around the collar to yield appropriate constriction. None of the CCA-transplanted mice in which no collar was placed developed lesions in the grafted artery apart from a small mural thrombus in one.

In all other mice, advanced plaques had developed focally immediately proximal to the constrictive collar with an area of unaffected vessel wall separating the lesion from the proximal anastomosis site (Figure 5b). In plaques that had formed in apoE−/− artery segments grafted into eGFP−apoE−/− mice (n=4), not a single eGFP−SMαA− cell

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**Figure 3.** Hematopoietic stem cells do not contribute to plaque SMCs. a, Overview of aortic root plaque from eGFP−apoE−/− BM → apoE−/− transplanted mouse (32 weeks of age) exhibiting donor-derived eGFP+ foam cells (green) and the formation of a fibrous cap of recipient-derived SMαA− cells (red only). L indicates lumen; C, fibrous cap; F, foam cells; M, tunica media; A, tunica adventitia. Scale bar=200 μm. b, Higher magnification of the area demarcated in a. Fluorescence microscopy combined with DIC imaging (gray scale) to reveal tissue structure. Scale bar=50 μm. c-e, Further analysis of the area demarcated in b. With the superimposed DIC image, it is often possible to visualize cell boundaries that would otherwise escape detection by fluorescence microscopy. Arrows indicate two separate cellular structures appearing as depressions in the relief-like DIC image (c), one of which stains positive for SMαA− (d, red channel). The neighboring cell is eGFP+ (e, red and green channels). No eGFP+ double-positive cells are present. Scale bar=25 μm.

**Figure 4.** Sequential immunostaining and FISH technique revealed no male SMαA− cells in plaques in male eGFP−apoE−/− BM → female apoE−/− transplanted mice. The figure shows analysis of an aortic root plaque from a mouse 20 weeks of age. a, SMαA immunostained sections were analyzed and the digitized images were stored. Deconvoluted optical section is shown. Arrows indicate nuclei that proved to contain a Y chromosome with subsequent FISH. Red indicates SMαA; Green, eGFP; Blue, DAPI. Scale bar=50 μm. b, FISH for Y chromosome. Fluorescence of eGFP is completely extinguished by the FISH procedure, and only the green fluorescence resulting from the FITC-conjugated Y chromosome paint probe is visible. Deteriorated SMαA− staining (red channel) is not shown. The phenotype of cells with Y+ nuclei is identified by comparing with the stored image (a). c-e, Larger magnifications of a and b. Scale bar=25 μm. F indicates foam cells; C, Fibrous cap; L, lumen.
First, we attempted and failed to reproduce the previous finding that hematopoietic stem cells can contribute to plaque SMCs in BM chimeric apoE−/− mice.7 Second, we directly evaluated and confirmed that plaque SMCs are derived from cells of the local vessel wall in a novel model of collar-induced atherosclerosis in surgically transferred artery segments.

**Figure 5.** Schematic of the model of constrictive collar-induced atherosclerosis in surgically transferred CCA segments. First, the graft was interpositioned in the recipient CCA by two end-to-end anastomoses. Then after 6 weeks, a slightly constrictive piece of silicone tubing was positioned around the distal part of the graft, and this induced the formation of an atherosclerotic plaque in the grafted CCA immediately proximal to the collar in the course of 10 weeks. b, Atherosclerotic plaque in the transplanted CCA 10 weeks after collar insertion. PA indicates proximal anastomosis; C, Collar; DA, distal anastomosis; P, plaque. Scale bar=1 mm.

**Discussion**

In this study, we traced the origin of plaque SMCs in different models of atherosclerosis in the apoE−/− hyperlipidemic mouse. First, we attempted and failed to reproduce the previous finding that hematopoietic stem cells can contribute to plaque SMCs in BM chimeric apoE−/− mice.7 Second, we directly evaluated and confirmed that plaque SMCs are derived from cells of the local vessel wall in a novel model of collar-induced atherosclerosis in surgically transferred artery segments.

**Origin of SMCs in Different Vascular Disease Models**

The present study is only one of several recent studies to have (re)investigated the origin of lesional SMCs in arterial disease, and not the first to have reached a conclusion that conflicts with others. Surprisingly few groups have examined the most prevalent arterial disease, atherosclerosis,7,16 but analogous investigations have been carried out in a number of other vascular disease models.8,9

The major discussion stemming from these studies has been over the question whether lesional SMCs can originate from BM cells. Besides the report of Sata et al on atherosclerosis in apoE−/− mice,7 this has been described to occur in human atherosclerosis,16 and in rodent models of wire injury,19 ferric chloride injury,21 and allotransplantation arteriopathy.7,22 Others, however, have not been able to detect BM-derived SMCs in allotransplantation arteriopathy,12 and it has been reported not to be involved in the pathogenesis of vein graft atherosclerosis,23 ligation-induced neointima,19 and neointima formed within a loose cuff.19

It is conceivable that part of the disparity in these findings, including the conflict between our results and those of Sata et al,7 is attributable to methodological or interpretational differences. For instance, it is strikingly difficult, despite a considerable literature, to find compelling images of BM-derived SMαA+ cells with the expected SMC morphology. But it is also reasonable to think that biological differences between models are important. Even though intimal SMC accumulation is the common hallmark of many types of vascular disease, the etiology and pathogenesis of lesion development differ. Interestingly, recruitment of circulating smooth muscle progenitor cells has predominantly been reported in models with significant endothelial disruption and platelet deposition, and experimental evidence indicates that SDFα expressed by adhering platelets facilitates homing of circulating progenitor cells.24 This hypothesis offers a possible explanation for the discrepancy between our results and those reported for coronary atherosclerosis in sex-mismatched BM transplanted patients.16 It is possible that asymptomatic plaque rupture with mural thrombus in humans, which is not seen in the mouse model, is critical in mediating homing of circulating progenitor cells.
The finding of intimal SMCs that do not originate from the graft or from hematopoietic stem cells in rodent models of vein graft atherosclerosis and allotransplantation arteriopathy has led to the theory that circulating smooth muscle progenitor cells of nonhematopoietic origin exist and participate in vascular lesion formation.\(^{25}\) It is, however, important to realize that migration of SMCs from the contiguous vasculature into the graft in those types of studies has not been conclusively excluded. For instance, Hu et al found that 40% of SMCs in atherosclerotic lesions of vein grafts were not derived from the local vessel wall or from hematopoietic stem cells, but the experimental design did not allow to distinguish between SMCs migrating through the anastomosis sites and homing and differentiation of SMCs from nonhematopoietic circulating progenitor cells.\(^{23}\) If migrating medial SMCs were the source of these cells, then the fact that our lesions developed isolated from the recipient vasculature by a stretch of unaffected donor vessel (Figure 6a), whereas lesions in the vein graft model did not, may explain the differences in the result obtained. Another explanation could be the inherent differences between SMCs of arterial and venous origin.\(^{26}\)

**Local Source of Plaque SMCs**

Our experiments were not designed to discriminate between candidate sources for plaque SMCs within the vascular wall. Several alternatives to vascular SMCs have been proposed, including endothelial-to-SMC differentiation and invasion of fibroblasts or stem cell progeny from the tunica adventitia.\(^{27,28}\) However, the identification of the local arterial wall as the origin of plaque SMCs established in this study combined with the observation of Feil et al that a major fraction of plaque SMCs originate from SM22\(^{\alpha}\) cells,\(^{6}\) pinpoint that in mice—that have no resident intimal SMCs—tunica media is the major contributor to SMCs in atherosclerotic plaques.

**Conclusions**

Our experiments demonstrate that atherosclerotic plaque SMCs are derived exclusively from the local vessel wall in apoE\(^{-/-}\) mice. This observation strongly supports the original hypothesis that these cells originate from local vascular SMCs and disagrees with the proposed role of circulating smooth muscle progenitor cells in atherogenesis. These findings have implications for future research into the mechanisms by which the fibrous component of atherosclerosis develops.

**Acknowledgments**

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Disclosures
None.

References
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Supplementary Methods

Transgenic mice

ApoE^/- mice (Taconic M&B, Ry, Denmark) and eGFP^+ mice (Jackson Laboratories, Bar Harbor, ME), both backcrossed more than ten times to C57BL/6 mice, were intercrossed to obtain eGFP^+apoE^-/- mice (hemizygous for the eGFP transgene).^1^ Genotyping for apoE was performed using primers recommended by the Jackson Laboratory (5-GCCTAGCCGAGGGAGAGCCG-3, 5-TGTGACTTGGAGCTCTGCAGC-3, and 5-GCCGCCCCGACTGCTCATCTCT-3). Phenotyping for eGFP was done using a Woods UV lamp.

Bone marrow transplantations

Crude bone marrow was flushed from femurs and tibias from eGFP^+apoE^-/- mice 8 weeks of age (n=7). Cells were washed twice in RPMI-1640 (Invitrogen) and filtered through a 70 μm mesh. ApoE^-/- mice (n=28) 8 weeks of age were irradiated with 10 Gy from a ^137^Cs source and 10^7^ unfractionated bone marrow cells were injected into a tail vein 4 hours later. Mice were housed with filter tops and administered oxytetracyclin (2g/l Terramycin vet. 20%) one day prior to and 7 days after transplantation.

Flow cytometry

Blood (50 μl) was lysed in erythrocyte lysis buffer (NH_4Cl 8.02 g/L, NaHCO_3 0.84 g/l, Na_2EDTA 0.37 g/l). The fraction of green fluorescent leukocytes in peripheral blood was measured in a forward-side scatter gate that was enriched for CD45^+ cells (>98%) and excluded only a few CD45^+ cells (~2%) as established in pilot experiments.
**Plasma lipids analysis**

Plasma total cholesterol and triglycerides were measured on a Vitros 950 analyzer (Ortho-Clinical Diagnostics). HDL cholesterol (HDL-C) was measured enzymatically on a Kone 30 analyzer (Thermo) using kits from ABX (Triolab, Copenhagen, Denmark).

**Common carotid artery segment transplantation and collar placement**

Donor common carotid arteries (CCA) were flushed with 100 IU/ml heparin saline and kept in RPMI-1640 at room temperature. Recipient mice were anesthetized with isoflurane (induction 5%, maintenance 1.5%-2%) and the right CCA was accessed through a midline neck incision. Heparin was administered (200 IU/kg i.m.) before microvascular clamps were applied. The recipient CCA was then divided and the donor CCA segment was interposed by two end-to-end anastomoses of four symmetrically placed 11-0 polyamide single sutures (Ethicon, Johnson & Johnson, Birkerod, Denmark). Total operation time was 75-90 minutes. Perioperative mortality was 15 per cent. Postoperative analgetics (buprenorphine, 0.1 mg/kg s.c. repeated every 12 hours for 3 days) were administered. An instructional video of the procedure can be obtained from the corresponding author.

Six weeks after CCA transplantation, mice were reoperated under isoflurane anesthesia and collars made from 0.75 mm silicone tubing (inner diameter 0.31 mm, HelixMark, Helix Medical Inc., CA, USA) were positioned around the most distal segment of the graft and secured by a single 7-0 ligature.

**Tissue processing**

Anesthetized mice (pentobarbital 5 mg i.p.) were killed by exsanguination, pressure-fixed with a phosphate-buffered (pH 7.2) formaldehyde solution (4%) through the left ventricle for 5 minutes,
and immersion-fixed for 6 hours at room temperature. The aortic root, aortic arch, right CCA, brachiocephalic trunk, abdominal aorta and descending thoracic aortas were removed, cryoprotected in a sucrose solution (25 % w/v for 24 h + 50 % w/v for 24 h), embedded in O.C.T\textsuperscript{TM} compound (Sakura Finetek, Pro-Hosp, Vaerloese, Denmark), and snap-frozen in liquid nitrogen-chilled methanol:acetone (1:1). Specimens were sectioned at 4-5 μm thickness.

**Immunohistochemistry**

SMCs were identified by staining for smooth muscle α-actin (SMαA) using biotinylated mouse monoclonal anti-SMαA (1:50, Clone 1A4, Neomarkers MS-113-B, AH Diagnostics, Aarhus, Denmark) followed by Alexa 594-conjugated streptavidin (1:200, Molecular Probes, Invitrogen, Taastrup, Denmark). Endogenous biotin was blocked with an avidin-biotin blocking kit (Vector Labs, VWR, Albertslund, Denmark) and a biotinylated irrelevant monoclonal antibody (1:50, Neomarkers NC-1390-B) of the same isotype was used as negative control. We would expect our two-layer immunofluorescence technique to be at least as sensitive as the one-layer method used by Sata et al.\textsuperscript{3} To further ensure sufficient sensitivity of our staining procedure, we initially compared this method to SMαA staining visualized by high-sensitive tyramide signal amplification (TSA\textsuperscript{TM} Biotin System, Perkin Elmer, Hvidovre, Denmark), and found that both techniques gave similar results in terms of number of SMαA\textsuperscript{+} cells identified (data not shown).

The Mac2 epitope was stained as a marker for plaque macrophages by using rat anti-mouse Mac2 antibody (1:500, CL8942AP, Cedarlane Labs, Trichem Aps, Frederikssund, Denmark) followed by Texas Red-conjugated goat anti-rat secondary antibody (1:200, Jackson Immunoresearch, Cambridgeshire, UK). An irrelevant rat monoclonal antibody (1:50, R2a00, Caltag, Trichem) was used as negative control.
**Fluorescence in situ hybridization (FISH)**

The Y chromosome was visualized in sections by a FITC-conjugated chromosome paint probe (Cambio, Cambridge, UK). Sections were first immunostained for SMαA, and high-power fields of SMαA+ cells were photographed. Then, the cover slips were removed and the sections were pretreated in 10 mM sodium citrate, pH 6.0 (2h, 80°C) (Bie & Berntsen, Aarhus, Denmark), digested in pepsin solution (0.025%, 10 min, 37 °C, Sigma), fixed in 4% paraformaldehyde and treated in freshly prepared acetic acid (0.25% acetic anhydride in 0.1 M triethanolamine-HCl, pH 8.0, 10 min, room temperature (Sigma)) to reduce unspecific binding of the probe. Two μl paint probe was applied to dehydrated sections under cover slips, and probe and tissue were denatured at 60 °C for 10 min followed by overnight hybridization at 37 °C. Slides were washed in 50% formamide/1xSSC (15 min, 37°C) and in 2xSSC (15 min, 37°C), before incubation in DAPI solution and mounting in Slowfade Light Antifade.

**Supplementary References**


Supplementary Table I. Plasma lipids in bone marrow transplanted mice at sacrifice

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<th>Total cholesterol mmol/L</th>
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<td>20 weeks of age</td>
<td>20.3±6.6</td>
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<td>(n=11)*</td>
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<td>32 weeks of age</td>
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<td>0.79±0.31</td>
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<td>(n=12)†</td>
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*On high fat diet. †On chow diet.
Supplementary Figure I

Supplementary Figure I. Not a single plaque SMC derived from donor bone marrow cells administered at bone marrow transplantation was found. Two high power fields of an aortic root plaque from an eGFP⁺apoE⁻/⁻ BM → apoE⁻/⁻ transplanted mouse (32 weeks of age) are shown. All SMαA⁺ cells are eGFP⁺. Color channels are shown separately [SMαA in (a,d), eGFP in (b,e) and combined (c,f)] to facilitate interpretation. L. Lumen. C. Fibrous cap. F. Foam cells. M. Tunica media. Red, SMαA. Green, eGFP. Greyscale, differential interference contrast. Scale bar 50 μm.
**Supplementary Figure II**

Collar-induced atherosclerosis in transplanted common carotid artery (CCA) segments is pathoanatomically reminiscent of spontaneously developed atherosclerosis in apoE\(-/-\) mice. In this figure the occurrence of foam cells is shown in an eGFP\(^+\) apoE\(-/-\) CCA graft anastomosed into an apoE\(-/-\) recipient mouse. As expected, foam cells in the plaque are derived from the circulation (eGFP\(^-\)) and positive for the macrophage marker Mac2. Red, Mac2. Green, eGFP. L, lumen, C. fibrous cap. F, foam cells. Scale bar 50 \(\mu\)m.