NG2 Proteoglycan Ablation Reduces Foam Cell Formation and Atherogenesis via Decreased Low-Density Lipoprotein Retention by Synthetic Smooth Muscle Cells

Zhi-Gang She, Yunchao Chang, Hong-Bo Pang, Wenlong Han, Hou-Zao Chen, Jeffrey W. Smith, William B. Stallcup

Objectives—Obesity and hyperlipidemia are critical risk factors for atherosclerosis. Because ablation of NG2 proteoglycan in mice leads to hyperlipidemia and obesity, we investigated the impact of NG2 ablation on atherosclerosis in apoE null mice.

Approach and Results—Immunostaining indicates that NG2 expression in plaque, primarily by synthetic smooth muscle cells, increases during atherogenesis. NG2 ablation unexpectedly results in decreased (30%) plaque development, despite aggravated obesity and hyperlipidemia. Mechanistic studies reveal that NG2-positive plaque synthetic smooth muscle cells in culture can sequester low-density lipoprotein to enhance foam-cell formation, processes in which NG2 itself plays direct roles. In agreement with these observations, low-density lipoprotein retention and lipid accumulation in the NG2/ApoE knockout aorta is 30% less than that seen in the control aorta.

Conclusions—These results indicate that synthetic smooth muscle cell–dependent low-density lipoprotein retention and foam cell formation outweigh obesity and hyperlipidemia in promoting mouse atherogenesis. Our study sheds new light on the role of synthetic smooth muscle cells during atherogenesis. Blocking plaque NG2 or altering synthetic smooth muscle cells function may be promising therapeutic strategies for atherosclerosis.

Key Words: atherosclerosis ■ chondroitin sulfate proteoglycan 4 ■ foam cells ■ macrophages ■ myocytes, smooth muscle

Elevated serum levels of low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) cholesterol are sufficient to drive the development of atherosclerosis in both humans and experimental animals, even in the absence of other known risk factors.1 Obesity, an independent risk factor for coronary heart disease mortality,2 is also associated with accelerated coronary atherosclerosis in both adolescent and young adult men.3 Although several cell types have been identified in mature atherosclerotic plaques, their detailed roles (especially roles of smooth muscle cells [SMCs]) in these phenomena and in the overall pathobiology of atherosclerosis4 are still unclear.

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Retention of apoB-containing lipoproteins (in particular LDL) in the subendothelial space is the key initiating process in atherogenesis.5–7 Once sequestered, these proatherogenic molecules are easily modified, generally by oxidation and aggregation.8 Modified lipoprotein accumulation triggers an inflammatory response, followed by recruitment of inflammatory cells, formation of foam cells, proliferation and migration of SMCs, assembly of extracellular matrix, and vascularization of plaques, a cascade that promotes lesion progression.6 Although the chondroitin sulfate proteoglycans biglycan and versican have been reported to play important roles in LDL retention,9 other mechanisms of arterial LDL sequestration remain to be elucidated.

NG2 proteoglycan, also known as the melanoma proteoglycan or chondroitin sulfate proteoglycan-4, is a cell surface protein highly expressed by different types of immature progenitor cells, including oligodendrocyte progenitors,10,11 chondroblasts and osteoblasts,12,13 immature keratinocytes,14 SMC,15–17 microvascular pericytes,11,18 and arterial adventitial pericyte progenitors.18 With certain exceptions (adipocytes),19 NG2 is downregulated as progenitors undergo terminal differentiation.20,21 Ablation of NG2 in vivo results in deficits in the development of many progenitor cells types.14,22–24 Importantly, NG2 ablation impairs brown fat development and function, leading to increased levels of serum lipids, increased lipid storage, and increased white fat mass.19 Our
current research uses the high-fat diet, apoE null mouse model to determine whether these properties of NG2 carry over to a role in atherogenesis.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Increased Plaque Expression of NG2 During Atherogenesis

NG2 is expressed highly in mouse embryonic dorsal aorta (E10.5), but it is downregulated in adult mouse aorta and major arteries (Figure 1A). Some NG2 expression persists in the adult ascending aorta, an area that is more prone to the development of advanced atherosclerosis. This suggests a potential role for NG2 in plaque formation.

Figure 1. Expression of NG2 proteoglycan in aorta and atherosclerotic plaques. A, Immunostaining of NG2 proteoglycan in embryonic (E10.5) dorsal aorta and different parts of aorta or artery of adult wild-type (WT) C57Bl/6j mice. Scale bar, 50 μm. B, Aortic sections at root level from male WT C57Bl/6j mice and apoE null mice after 0, 8, 16, and 26 wk (W) of high-fat diet feeding were immunolabeled with rabbit anti-NG2 antibody. The scale bars are 200, 100, and 50 μm in the top, middle, and bottom, respectively. C, The abundance of NG2 proteoglycan was quantified by calculating the ratio between NG2-positive area and plaque or media area. n=3 in each group. L indicates lumen; M, media; and P, plaque.
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of atherosclerotic plaques (Figure 1A). When compared with aortas without plaques (from wild-type mice and apoE null mice on regular chow), plaques in aortas from apoE null mice on the high-fat diet exhibit increasing levels of NG2 expression at 8, 16, and 26 weeks of induction, eventually comprising 10% of the entire plaque area (Figure 1B). When compared with wild-type aorta, NG2 is more highly expressed in the media of apoE null aorta although this gradually decreases as atherosclerosis progresses (Figure 1B). During a 16-week induction period, NG2 expression becomes abundant in plaques throughout the aortic tree, including the aortic root, brachiocephalic artery, carotid artery, aortic arch, thoracic aorta, and abdominal aorta (Figure I in the online-only Data Supplement). Judged by comparison with labeling of nuclei, the majority of plaque NG2 expression is cell associated although some NG2 is shed from cell surfaces and associates with the extracellular matrix. Double immunolabeling studies demonstrate that NG2 is absent from CD31-positive endothelial cells and from α-smooth muscle actin–positive contractile SMCs (c-SMCs; Figure 2) and is associated with 17.7% of CD68-positive macrophages (Figure 2). On the contrary, ≈80% of NG2-positive plaque cells express the synthetic SMC (s-SMC) marker nonmuscle myosin heavy chain-β (Figure 2). NG2-expressing cells are also positive for platelet-derived growth factor receptor receptor-β (100%; Figure 2), which has been reported as a marker for mural cells (supporting pericytes and vascular SMCs), pericyte progenitors/mesenchymal stem cells, and myofibroblasts. These results indicate that NG2 is primarily expressed in plaques by s-SMCs that may also exhibit progenitor cell characteristics during atherogenesis.

NG2 Ablation Aggravates High-Fat Diet–Induced Obesity and Hyperlipidemia in ApoE Null Mice

To explore the potential role of NG2 in atherogenesis, male apoE null/NG2 null and apoE null mice were fed with a high-fat diet for 10, 16, and 26 weeks to induce early, intermediate, and late-stage atherosclerotic plaques. On this regimen, male apoE null/NG2 null mice gain more weight than apoE null mice (Figure 3A), a difference that becomes statistically significant after 26 weeks of high-fat diet induction. At this time point, male apoE null/NG2 null mice weigh 27.5% more than their apoE null counterparts (Figure 3A) and have epididymal fat deposits that are twice as large as those in apoE null mice (Figure IIA in the online-only Data Supplement). Hematoxylin-eosin staining of epididymal fat pad sections reveals that white adipocytes in apoE null/NG2 null mice are >2-fold larger in cross-sectional area than white adipocytes in apoE null mice (Figure IIB in the online-only Data Supplement), indicative of increased lipid storage. Comparison of serum lipid levels between apoE null/NG2 null and apoE null mice after 26 weeks of high-fat diet feeding reveals that the double null mice have significantly increased levels of total cholesterol (71%) and triglycerides (72.1%) (Figure 3B and 3C). Differences in lipid levels are not statistically significant at early and intermediate stages of atherogenesis. Gel filtration of pooled plasma samples demonstrates that

Figure 2. Identity of NG2-positive cells in mouse atherosclerotic plaque. Aortic sections at root level were costained with NG2 antibody and other antibodies labeling endothelium (CD31), macrophages (CD68), contractile smooth muscle cells (smooth muscle actin [SMA]), synthetic smooth muscle cells (β-myosin heavy chain [MHC]), and progenitor cells (platelet-derived growth factor receptor receptor-β [PDb]). Right-hand panel (scale bar=10 μm) is high-power image of the square in the left-hand panel (scale bar=50 μm). The percentage of NG2-expressing cells expressing other markers is determined from these double labeling studies. n=5 in each staining. L indicates lumen; M, media; and P, plaque.
levels of VLDL in apoE null/NG2 null mice are 2-fold higher than in apoE null mice, whereas plasma LDL, intermediate density lipoproteins, and HDL levels are similar between the 2 groups (Figure 3D). These results indicate that proatherogenic VLDL, but not HDL, accounts for the higher total cholesterol seen in apoE null/NG2 null mice. Plasma glucose levels are similar between apoE null/NG2 null mice and apoE null mice (Figure IIIA in the online-only Data Supplement), and no significant difference is found in glucose tolerance between the 2 groups (Figure IIIB in the online-only Data Supplement). These findings are consistent with our published data on apoE wild-type, NG2 null mice,19 showing that NG2 ablation also aggravates obesity and hyperlipidemia in apoE null mice.

NG2 Ablation Inhibits High-Fat Diet–Induced Atherogenesis in apoE Null Mice

The extent of plaque progression (plaque area/aortic surface area) was then determined in apoE null and apoE null/NG2 null mice after 10, 16, and 26 weeks of high-fat diet induction. Although plaque areas are similar during early and intermediate stages of atherogenesis, a 30% reduction in plaque area is evident in male apoE null/NG2 null mice at 26 weeks of induction (Figure 4A).

Plaque areas were also measured by morphometry in hematoxylin-eosin–stained transverse sections of the aortic root after 16 and 26 weeks of induction. Plaque size is again found to be reduced by ≈30% after 26 weeks of high-fat diet induction in the double knockout mouse although atherogenesis was similar between the 2 groups after 16 weeks of induction (Figure 4B). The extent of the necrotic core of the plaques is similar between the 2 groups of mice (Figure IV in the online-only Data Supplement). Plaques in NG2 null/ApoE null mice exhibit 20% to 30% reductions in lipid deposition, collagen content, macrophage abundance, and numbers of both contractile SMCs and s-SMCs18,27,28 (Figure 5A and 5B). The relative abundance (ratios) of the major plaque components is

**Figure 3.** NG2 ablation aggravates high-fat diet-induced obesity and hyperlipidemia in apoE null mice. A, Body weight of male apoE null and apoE null/NG2 null male mice (n=15–18) after 10, 16, and 26 wk (W) of high-fat diet feeding. B, Plasma cholesterol levels in male apoE null and apoE null/NG2 null male mice (n=15–18) after 10, 16, and 26 W of high-fat diet feeding. C, Plasma triglyceride levels in male apoE null and apoE null/NG2 null male mice (n=15–18) after 10, 16, and 26 W of high-fat diet feeding. D, Gel filtration analysis of plasma lipoproteins from male apoE null and apoE null/NG2 null male mice (n=15–18) after 26 W of high-fat diet feeding. *P<0.05, **P<0.01, and ***P<0.001. WT indicates wild-type.
similar between the 2 groups, except that plaques in the double knockout mice exhibit increased collagen deposition (Figure V in the online-only Data Supplement). The calculated plaque stability score for plaques in the NG2 null group is increased when compared with those in the control group although not to a significant degree (Figure 5C). In female apoE null mice, NG2 ablation does not affect body weight (Figure VI in the online-only Data Supplement) but still results in reduced aortic plaque burden (Figure VII in the online-only Data Supplement) after 26 weeks of high-fat diet feeding.

**s-SMCs Sequester LDL and Promote Foam Cell Formation by Macrophages**

Reduced atherogenesis in apoE null, NG2 null mice is counterintuitive in light of the increased obesity and hyperlipidemia observed in these mice. To explore mechanisms that underlie this surprising phenotype, the NG2-expressing plaque cell population was expanded from mouse atherosclerotic plaque explants. In culture, these cells exhibit the same characteristics as NG2-expressing plaque cells in situ (Figures VIII and IX in the online-only Data Supplement). We found that cultured plaque s-SMCs sequester fluorescently labeled LDL in a concentration-dependent manner (Figure 6A) and also bind acetylated LDL (Ac-LDL; Figure 6B), a stable mimic of oxidized LDL. Binding of LDL to s-SMCs did not affect cell viability (Figure X in the online-only Data Supplement) or differentiation to contractile SMCs (Figure XI in the online-only Data Supplement). In vivo, although LDL mostly accumulates in plaque macrophages, a close association of LDL with NG2-positive s-SMCs is also observed in some areas of mouse plaques (Figure 6C). The magnitude of BODIPY-LDL sequestration by s-SMCs in vitro was similar when the lipid was diluted in either PBS or hypo-K⁺ solution and then incubated with plaque s-SMCs (Figure 6D). Because hypo-K⁺ solution has the documented ability to inhibit lipid membrane internalization, this result indicates that LDL retention by s-SMCs occurs at the cell surface rather than via internalization. Plaque s-SMCs also sequester triglyceride-rich lipoproteins such as VLDL (Figure XII in the online-only Data Supplement), which are the main lipoproteins found to increase in apoE null/NG2 null mice (compared with apoE null mice). Even though extracellular matrix and purified proteoglycans have both been shown to sequester LDL and VLDL, the LDL and VLDL-sequestering capability of cultured s-SMCs was dramatically greater than that of the extracellular matrix produced by these cells (Figure XII in the online-only Data Supplement). Triglyceride-rich lipoproteins such as VLDL are unlikely to interact with intraplaque s-SMCs unless they are lypolyzed because these large particles are unable to directly enter the arterial wall. Hence, we focused on LDL in subsequent experiments. Overall, these findings indicate that plaque s-SMCs may be important for sequestering LDL.

The interaction of s-SMCs with LDL suggests that s-SMCs might be able to promote foam cell formation by presenting LDL to macrophages. To test this possibility, we added RAW264.7 cells to wells containing Ac-LDL with or without plaque s-SMCs. In wells containing plaque-derived s-SMCs, Ac-LDL-dependent (20 µg/mL) foam cell formation is enhanced in proportion to the number of added s-SMCs. As a control, s-SMCs themselves do not generate foam cells in Ac-LDL–containing medium (Figure 7A). At higher Ac-LDL levels (200 µg/mL), the stimulatory effect of plaque s-SMCs on foam cell formation is less robust (Figure XIIIA in the online-only Data Supplement), suggesting that s-SMC contributions to foam cell formation are more important when Ac-LDL levels are relatively low. When RAW264.7 cells are cultured in Ac-LDL–containing medium (20 µg/mL) conditioned overnight by confluent plaque s-SMCs, the extent of foam cell formation is not enhanced relative to that of RAW264.7 cells cultured in Ac-LDL–containing medium conditioned overnight by confluent RAW264.7 cells (Figure 7B). This result indicates that physical contact between macrophages and plaque s-SMCs, rather than soluble s-SMC–derived factors, is required to facilitate foam cell formation. In addition, phagocytosis of Dil-Ac-LDL by RAW264.7 cells is enhanced by the presence of plaque s-SMCs in proportion to the number of added plaque s-SMCs (Figure 7C), verifying that lipid accumulation by macrophages is the result of Ac-LDL uptake from the medium, rather than enhanced lipid synthesis by macrophages. In contrast, endothelial cells (human umbilical vein cells), also known to bind
Ac-LDL,33 do not promote RAW264.7 phagocytosis of Dil-Ac-LDL (Figure XIIIB in the online-only Data Supplement), demonstrating the specificity of the s-SMC effect. Similar to RAW264.7 macrophages, phagocytosis of Dil-Ac-LDL by primary mouse peritoneal macrophages is also enhanced by the presence of plaque s-SMCs (Figure 7D).

NG2 Mediates LDL Binding and s-SMC–Dependent Macrophage Conversion to Foam Cells

The fact that the chondroitin sulfate proteoglycans biglycan, versican, and perlecan have been implicated in arterial retention of LDL,9,31 suggests the possibility that the NG2 chondroitin sulfate proteoglycan might mediate LDL binding to
s-SMCs. To investigate the LDL-binding capability of NG2, purified NG2 core protein, NG2 proteoglycan (Figure XIV in the online-only Data Supplement) or BSA (control) were coated in wells, followed by incubation with serial dilutions of human LDL. The amount of bound LDL was determined by sequential incubations with anti-LDL primary antibody and horseradish peroxidase–conjugated secondary antibody. The results indicate that both NG2 core protein and NG2 proteoglycan bind LDL in a concentration-dependent fashion, and that the binding capacity of NG2 is not affected by the presence or absence of the chondroitin sulfate chain (Figure 8A). To validate this, we compared the LDL-binding capacity of wild-type and NG2 null s-SMCs (both were enriched by cell sorting based on platelet-derived growth factor receptor receptor-β expression). Varying concentrations of fluorescently labeled human LDL were incubated with the 2 cell types. LDL binding, quantified by fluorescence intensity, was dramatically reduced in the case of NG2 null s-SMCs (Figure 8B). We then compared the ability of wild-type and NG2 null s-SMCs to facilitate macrophage conversion to foam cells. Foam cell formation by RAW264.7 macrophages was diminished in the presence of NG2 null s-SMCs when compared with the more robust foam cell formation seen in the presence of wild-type s-SMC (Figure 8C). Immunohistochemistry and Oil-Red-O staining of aortic root sections after 26 weeks of atherosclerosis induction indicates that LDL accumulation (Figure 8D) and lipid accumulation in foam cells (Figure 5A and 5B) are significantly reduced in apoE null/NG2 null plaques when compared with that seen in the apoE null plaques. Expression levels of the lipoprotein-binding proteoglycans perlecan and biglycan were similar in the aortas of apoE null/NG2 null and apoE null mice after 26 weeks of high-fat diet induction (Figure XV in the online-only Data Supplement). These in vivo data support the role of NG2 in sequestering LDL during the development of atherosclerotic plaques and at least partly explain how NG2 ablation can reduce atherogenesis despite aggravating obesity and hyperlipidemia.

**Discussion**

Our results show that NG2 chondroitin sulfate proteoglycan expression, primarily by s-SMCs, progressively increases in plaques during atherogenesis in apoE null mice. Ablation of NG2 leads to significantly reduced plaque development, even in the face of aggravated obesity and hyperlipidemia in these mice.

In mouse atherosclerotic plaque, well-recognized populations of plaque cells (endothelial cells, contractile-SMC, and macrophages) are largely negative for NG2 expression. Instead, NG2 is primarily expressed in developing plaques by nonmuscle myosin heavy chain isoform-B–positive s-SMCs. These cells also express the platelet-derived growth factor receptor receptor-β marker that characterizes progenitor cells in other tissues, including the aortic wall, suggestive of a progenitor phenotype for plaque NG2-expressing plaque cells. Because progenitors in normal tissues rarely represent >1% of the total cell population, a novel aspect of our research is the surprising abundance of progenitor-like s-SMCs in atherosclerotic plaques. This may reflect the ability of progenitor-like cell populations to expand significantly in response to injuries and other pathologies. For example, pericyte progenitors increase significantly after arterial injury, to the extent that they contribute to restenosis during arterial repair. We find that the plaque s-SMC population expands during atherogenesis to comprise ~10% of the plaque cell population, suggesting a potentially important contribution of these cells to atherogenesis. In this respect, our results demonstrate a role for s-SMCs in promoting foam cell formation. Because macrophage-derived foam cells are the major repository of cholesterol esters in plaques, and because foam cell formation
is the hallmark of both early and late atherosclerotic lesions, the ability of plaque s-SMCs to promote foam cell formation identifies a pivotal role for these cells in plaque development.

In exploring mechanisms underlying s-SMC–enhanced foam cell formation, we find plaque s-SMCs can sequester both LDL and modified LDL. Retention of apoB-containing lipoproteins in the subendothelial space, often attributed to the proteoglycan-rich subendothelial extracellular matrix, is a key step in initiating atherogenesis. Sequestered proatherogenic molecules are readily modified by oxidation and aggregation to yield inflammatory products that activate endothelial, smooth muscle, and inflammatory cell signaling. This promotes continued lesion development by stimulating recruitment of additional monocytes/macrophages. The ability of s-SMCs to sequester LDL suggests that s-SMCs can be important in presentation of LDL to phagocytic cells. In contrast, although endothelial cells have also been reported to bind Ac-LDL, human umbilical vein endothelial cells do not promote foam cell formation by macrophages in our assays. This emphasizes the unique nature of s-SMCs as promoters for foam cell formation. Our results suggest that plaque s-SMCs may play an increasingly important role in LDL retention as plaque development progresses because the s-SMC population expands with plaque growth.
that purified NG2 binds LDL, and that wild-type s-SMCs bind LDL more effectively than NG2 null s-SMCs. Moreover, ablation of NG2 diminishes the ability of s-SMCs to promote foam cell formation by macrophages in vitro. In contrast to the binding of LDL to extracellular matrix proteoglycans,9,29 the interaction between LDL and NG2 is associated with the s-SMC cell surface because NG2 is mainly expressed on the surface of s-SMCs in plaques. This demonstrates not only the important role of NG2 in LDL binding but also the novel role of s-SMCs in LDL retention and foam cell formation. Although the functions of many other proteoglycans is mediated mainly by their glycosaminoglycan chains, the NG2 core protein binds LDL without any requirement for the chondroitin sulfate chain. This property makes NG2 unique from other proteoglycans in terms of LDL binding. The NG2 core protein is also responsible for binding several other types of ligands, including kringle domain proteins,40 integrins,40 type VI collagen,40 membrane type-1–matrix metalloproteinase,41 and growth factors.42,43 For example, the NG2 core protein has been shown to bind fibroblast growth factor-142 and platelet-derived growth factor-AA,43 2 growth factors that may affect atherogenesis.44,45 As a chondroitin sulfate proteoglycan, NG2 may also bind chemokines, which are also critical for atherogenesis.46 It remains to be determined whether interference with these interactions contributes to the reduced atherogenesis caused by NG2 ablation.

To investigate the importance of NG2 in atherogenesis, we compared apoE null/NG2 null mice with apoE null mice for progression of atherosclerotic plaque formation. After 26 weeks of high-fat diet induction, plaque area is reduced by 30% in the apoE null/NG2 null mice. This difference is not observed at 10 and 16 weeks, possibly because the size of the s-SMC population and the associated accumulation of NG2 in plaques is not substantial enough to exert an effect at these early time points. This result supports the putative involvement of NG2 as a component of s-SMCs responsible for LDL accumulation and foam cell formation, both of which are significantly reduced in apoE null/NG2 null mice compared with apoE null mice. Interestingly, reduced plaque formation in apoE null/NG2 null mice occurs in spite of the obesity and...
hyperlipidemia that develop in these germline NG2 null animals. The double null mice exhibit a 2-fold increase in plasma levels of VLDL, a cholesterol species responsible for driving arterogenesis in both humans and experimental animals, even in the absence of other known risk factors. Obesity is also associated with accelerated coronary arterogenesis.3,48 Our American Heart Association fellowship 10POST3770077 to Dr She, HL80718 to Dr Smith and RO1 CA95287 to Dr Stallcup, and by This work was supported by National Institutes of Health grants UO1 approach to verify our hypothesis if an s-SMC–specific Cre mouse can be identified. In summary, we have detected robust expression of NG2 proteoglycan by progenitor-like s-SMCs during arterogenesis. Plaque s-SMCs facilitate foam cell formation by interacting with LDL. NG2 on plaque s-SMCs mediates LDL retention, so that NG2 ablation leads to reduced LDL sequestration in the aorta and reduced arterogenesis, even in the face of increased plasma VLDL. Our work provides new information about the important roles of s-SMCs and NG2 in plaque development. The functions of plaque NG2 and s-SMCs in lipid retention and foam cell formation appear to outweigh NG2 roles in obesity and hyperlipidemia in determining the progression of mouse arterogenesis. Plaque NG2 inhibition along with therapeutic manipulation of plaque s-SMC development and function might be viable strategies for therapy of arterogenesis.

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Disclosures
None.

References
Significance

Accumulation of cholesterol-rich lipoprotein in the artery wall leads to atherosclerosis. The mechanisms underlying this sequestration in the aorta remain to be fully elucidated. Although several cell types (including smooth muscle cells) are recruited to developing plaques as a result of lipoprotein retention, the detailed roles of these cell types in the pathobiology of atherosclerosis are still unclear. Our research indicates that synthetic type smooth muscle cells can drive atherosclerosis via promotion of foam-cell formation by macrophages, a process that partly depends on NG2 proteoglycan-dependent sequestration of low-density lipoproteins. In spite of aggravated obesity and hyperlipidemia, NG2 null mice exhibit significantly decreased formation of atherosclerotic plaques. This sheds new light on the role of a subclass of smooth muscle cells during atherosclerosis. Blocking plaque NG2 or altering synthetic smooth muscle cell recruitment and function may be promising novel therapeutic strategies for treating atherosclerosis.
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SUPPLEMENTAL MATERIAL

NG2 ablation Reduces LDL Retention of Synthetic Smooth Muscle Cells and Atherogenesis

She, NG2 Ablation Reduces Atherogenesis

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Supplemental Tables

**Supplemental Table SI:** List of commercially available primary antibodies

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**Supplemental Table SII:** List of secondary antibodies used in current research

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**Supplemental Figures**

**Supplemental Figure I.** NG2 proteoglycan is present in mouse atherosclerotic plaque throughout the aorta after 16 weeks of high fat diet. Atherosclerotic plaque sections from aortic root (H-A, A), brachiocephalic artery (B-A, B), carotid artery (C-A, C), aortic arch (Arch, D), thoracic aorta (T-A, E), and abdominal aorta (A-A, F) were stained with H&E in the left-hand panel of each pair. Adjacent sections were immunolabeled for NG2 (green) in the right-hand panel of each pair. Scale bars: left-hand panel of A, D, E and F, 400 µm; left-hand panel of B and C, 200 µm; right-hand panels of all pairs, 50 µm. M, media; P, plaque; L, lumen.
Supplemental Figure II. NG2 ablation aggravates high-fat diet induced expansion of white fat tissue.  A. Dissected epididymal fat pads from male ApoE null and ApoE null/NG2 null mice (n=3) after 26 weeks (W) of western diet feeding. Quantification is shown in right panel. 
B. Representative cross sections of epididymal fat pads from male ApoE null and ApoE null/NG2 null mice after 26 weeks of western diet feeding. Quantification is (N=3) shown in right panel. *, p<0.05; **, p<0.01

Supplemental Figure III.  A. Plasma glucose levels of male ApoE null and ApoE null/NG2 null male mice (n=15-18) after 10, 16, and 26 weeks (W) of western diet feeding. B. Glucose tolerance testing of male ApoE null and ApoE null/NG2 null male mice (n=9 and 7) after 26 W of western diet feeding.
**Supplemental Figure IV.** Ratio of necrotic area in plaques of ApoE null and ApoE null/NG2 null after 26 weeks of western diet feeding. N=20-22.

**Supplemental Figure V.** Ratio of different components of plaque in the aorta of ApoE null and ApoE null/NG2 null after 26 weeks of western diet feeding. N=10. *, P<0.05.
**Supplemental Figure VI.** Body weight of female ApoE null and ApoE null/NG2 null mice (n=15-18) after 10, 16, and 26 weeks (W) of western diet feeding.

![Body Weight Graph](Path_to_graph)

**Supplemental Figure VII.** NG2 ablation inhibits western diet-induced atherogenesis in female ApoE null mice. Plaque areas of aortic atherosclerotic lesions in ApoE null and ApoE null/NG2 null (n=16-20) female mice were quantified as a function of aortic area, and compared after 10, 16, and 26 weeks (W) of western diet feeding.

![Plaque Area Graph](Path_to_graph)
Supplemental Figure VIII. Culturing NG2-positive s-SMCs from mouse plaque.
Atherosclerotic plaque-burdened mouse aortas (A) were dissected and opened longitudinally. Plaques (C) were peeled from the aortic wall (B) and minced into 0.5 mm pieces (D). Fibroblast-like cells migrate out of plaque fragments to form a monolayer over 10 days of in vitro expansion (D).
E. Plaque cells labeled with primary NG2 antibody and corresponding Alexa-488 conjugated secondary antibody were used for cell sorting. Plaque cells highly expressing NG2 were collected and maintained as NG2-positive plaque cells. L: lumen; P: plaque; W: aortic wall.
**Supplemental Figure IX.** Characterization of expanded NG2-expressing plaque cells. **A.** Staining for NG2 and SMA on cells expanded from isolated atherosclerotic plaque. **B.** Bright field image of NG2-high cells expanded from plaque. **C.** Flow cytometric analyses of NG2-high cells expanded from plaque.

**Supplemental Figure X.** Viability of s-SMCs after treatment with 50 µg/ml of LDL for three days.
Supplemental Figure XI. NG2 and SMA staining on s-SMCs after treatment with 50 µg/ml LDL for three days.

Supplemental Figure XII. Retention of LDL or VLDL by plaque s-SMCs or extracellular matrix deposited by plaque s-SMCs. Scale bar=20µm
Supplemental Figure XIII. Foam cell formation promoted by s-SMC or by HUVECs in response to Ac-LDL. A. Raw264.7 macrophages were induced to form foam cells by addition of 20 or 200 µg/ml Ac-LDL with or without 1.5x10^5 plaque s-SMCs. Efficiency of foam cell formation is quantified by dividing Oil Red O intensity by RAW264.7 cell number. B. Foam cell formation by macrophages was induced by addition of 20 µg/ml Dil-Ac-LDL to wells with or without 1.0x10^5 plaque s-SMCs or HUVECs. Efficiency of foam cell formation is quantified via dividing Dil fluorescence intensity by macrophage number. **: p<0.01; ***: p<0.001.

Supplemental Figure XIV. SDS-PAGE electrophoresis of DEAE-purified recombinant NG2 ectodomain. NG2-P1 and NG2-P2 represent the first and second pools of material from DEAE chromatography. Different amount of NG2-P1 (8, 16, and 32 µg in lane 2, 3 and 4) and NG2-P2 (6, 12, and 24 µg in lane 6, 7 and 8) proteins were run on a 6% gel. Gels were calibrated with globular molecular mass marker standards (indicated in left margin in kilodaltons). The double band for the core protein is a result of proteolysis.
Supplemental Figure XV. Expression of Biglycan and Perlecan in the aorta of ApoE null and ApoE null/NG2 null after 26 weeks of western diet feeding. RNA was extracted from the aorta of male ApoE null and ApoE null/NG2 null after 26 weeks of western diet feeding using Qiagen RNeasy Mini Kit. The expression level of Biglycan, Perlecan and internal control GAPDH were quantified via qPCR. Expression levels of Biglycan and Perlecan were normalized to GAPDH. N=3.
NG2 ablation Reduces LDL Retention of Synthetic Smooth Muscle Cells and Atherogenesis

Materials and Methods

Animals
NG2 deficient mice\(^1\) were crossed with Apolipoprotein E (ApoE) null C57Bl/6 mice (Jackson Laboratories) to obtain ApoE null/NG2 null mice. These mice and ApoE-null counterparts were maintained in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Sanford-Burnham vivarium. All animal procedures were performed in accordance with Office of Laboratory Animal Welfare Regulations and were approved by the Sanford-Burnham Institutional Animal Care and Use Committee prior to execution. Mice were fed normal chow for 8 weeks and then switched to high-fat diet (Teklad Adjusted Calories Western-type diet, 21% fat, 19.5% casein, and 0.15% cholesterol, TD 88137) to induce atherogenesis. Following induction, mice were euthanized, bled via the retro-orbital vein, and perfused successively with ice-cold PBS and 4% paraformaldehyde. Hearts, aortas and epididymal fat pads were collected for later analysis.

Antibodies
Polyclonal antibodies against Neuron-glia antigen 2 (NG2) ectodomain\(^2, 3\), platelet-derived growth factor receptor beta (PDGFR\(\beta\))\(^4, 5\) and NG2/D2\(^6\) have been previously described. Commercial antibodies are described in the online supplemental materials (supplemental Tables I and II).

Histology and microscopy
Mouse plaque tissue was prepared as described\(^7\). Tissue samples were frozen in OCT for preparation of serial 10 or 20 µm frozen sections. Immunolabeling of sections and analysis by confocal microscopy were performed as previously described\(^4, 5\). A BioRad inverted Radiance 2100 Multiphoton Confocal Microscope was used to obtain images. Control slides were used to establish exposure parameters. The specificity of NG2 antibodies has been established in multiple published reports. This was further verified by the absence of NG2 labeling in plaques from NG2 null mice. Specificity and absence of off-target labeling by second antibodies were established via omission of primary antibodies. Confocal images were processed with ImageJ software to obtain merged images for determining spatial relationships between markers. Z-stack serial images were processed using Volocity software, allowing unambiguous identification and quantification of plaque cell types in three-dimensional reconstructions.

Culture of Mouse Atherosclerotic Plaque Cells
After 16 weeks on the Western diet, ApoE null or ApoE null/NG2 null mice were anaesthetized with Avertin and transcardially perfused with cold PBS. Aortic trees were dissected and opened longitudinally. Plaques on the luminal surface of the aorta were collected under a dissecting microscope and minced into 0.5 mm pieces. Plaque fragments were placed in gelatin-coated (Sigma, G2500) wells and cultured in mesenchymal stem cell expansion medium (MSCEM, SCM015, Millipore). Over a period of 10 days, migratory cells from these fragments formed a monolayer on the culture surface.

Flow Cytometry
Analytical flow cytometry using cultured plaque s-SMCs was performed as previously described\(^8\), using the FACSCanto instrument. Preparative cell sorting to enrich for NG2-
positive cells was based on labeling of s-SMCs with rabbit antibody against NG2. For comparison of wild type and NG2 null s-SMCs, cells expanded from plaques were enriched for s-SMCs based on labeling with rabbit antibody against PDGFRβ. Wild type and NG2 null s-SMCs expressed similar levels of PDGFRβ, and a-smooth muscle actin (SMA), differing only in the presence or absence of NG2.

**Solid-phase LDL binding to NG2.**
96-well high-absorb plates were coated with 50 µg/ml NG2 core protein (NG2-P1), NG2 proteoglycan (NG2-P2) or BSA (control) in HBS buffer (20 nM HEPES, 150nM NaCl). Serially diluted (factors of two) human LDL was incubated in these wells, and bound LDL was detected with anti-LDL primary antibody and HRP-conjugated secondary antibody. After reaction with chromogenic substrate (TMB), the plates were read to evaluate the amount of LDL retained in the coated wells. Four replicate wells were used for each concentration of LDL to allow determination of an average value for binding. Overall results are presented as mean values (±SEM) from three independent experiments.

**Lipoprotein binding to s-SMCs**
Plaque s-SMCs in 8-well chamber slides were incubated for 1 hour at 37°C in DMEM containing 10% FCS and 0, 10, 30 or 100 µg/ml of BODIPY-LDL (Invitrogen, L-3483) or 30 µg/ml Dil-Ac-LDL (Biomedical Technologies Inc, BT-902). Cells were washed 3 times with DMEM/10% FCS and fixed with cold 4%PFA for 10 minutes. After permeabilization for 10 minutes with 0.1% Triton X-100, slides were mounted in DAPI-containing medium. To examine surface versus intracellular localization of LDL after incubation with s-SMCs, BODIPY-LDL (100 µg/ml) was diluted into PBS or Hypo-K solution (71 mM KCl, 1.8 mM CaCl₂, 10 mM Heps, 0.17 mM K₂HPO₄, 0.175 mM KH₂PO₄, and 0.405 mM MgSO₄, 150 mOsm) followed by a 1 hour incubation with s-SMCs at 37°C. Hoechst 33342 was added to visualize nuclei 20 minutes before confocal imaging.

**Binding of LDL and VLDL to s-SMC Extracellular Matrix Material**
Extracellular matrix material was prepared from s-SMCs as described. s-SMCs grown in wells were lysed in situ, followed by extensive washing of the wells. LDL and VLDL binding to these wells was compared with binding to wells containing living cells. Cell-free wells were used as background controls. BODIPY-LDL (Invitrogen, L-3483) or Dil-VLDL (Biomedical Technologies Inc, BT-922) were incubated with s-SMCs or extracellular matrix at concentrations of 100 µg/ml for 2 h at 37°C, followed by three washes with PBS. Fluorescence intensity was imaged under confocal microscopy as described above.

**Foam cell formation**
Plaque s-SMCs or human umbilical vein endothelial cells (HUVEC) were seeded in Lab-Tek II CC2 8-well glass chamber slides (154941) and allowed to recover for 4 hours. Medium was then replaced with 200µl of DMEM/10%FCS containing human Ac-LDL (0, 20, 200 µg/ml) (Biomedical Technologies Inc, BT-906) or Dil-Ac-LDL (Biomedical Technologies Inc, BT-902), followed by incubation for 2 hours at 37°C. Some wells were supplemented with 200 µl of DMEM/10%FCS containing 1×10⁵ RAW264.7 cells or mouse peritoneal macrophages (MPM), followed by additional 24 (For Ac-LDL) or 4 (For Dil-Ac-LDL) hour incubations. Wells without macrophages cells were used as controls. Slides were washed 3 times with PBS, and cells were fixed with 4% PFA for 10 minutes. After washing with PBS, Ac-LDL treated slides were treated with 60% isopropanol for 10 minutes and stained with 0.5% Oil Red O solution for 30 minutes. Slides were washed with water to obtain a clear background and mounted for bright field imaging. Dil-Ac-LDL
treated slides were treated with 0.1% Triton-X-100 for 5 minutes and mounted with DAPI containing medium. Confocal images were captured in 5 randomly selected fields in each well. Foam cell formation in each well was quantified by dividing the intensity of Oil Red O or Dil signals by macrophage number.

**Plaque Quantification and Histomorphometric Analysis**
En face aortic atherosclerotic lesions were analyzed as described\(^7\), \(^11\). Atherosclerotic plaques at the aortic valve level were sectioned and quantified based on hematoxylin/eosin (H&E) staining as described\(^7\), \(^12\). Plaque composition was assessed by staining with Oil red O (lipid-rich cores), Masson trichrome (collagen), anti–αSMA antibody (contractile smooth muscle cells), anti-PDGFRβ (synthetic smooth muscle cells), and anti–MOMA-2 antibodies (macrophages) as described\(^7\). Plaque stability was evaluated by comparing the plaque stability score PSC = (collagen + Smooth muscle cell area)/(Macrophage + Lipid area)\(^7\), \(^12\), \(^13\).

**Measurements of Plasma Cholesterol, triglycerides, glucose and Glucose Tolerance Test (GTT)**
Mouse plasma cholesterol, triglycerides, and glucose were measured by the Yale Mouse Metabolic Phenotyping Center. GTT was performed as described\(^3\).

**Statistical Analyses**
All data are expressed as mean ± SEM. Results were analyzed by one-way anova followed by post-hoc analysis for multiple comparisons or 2-tailed Student’s t test for comparing between two groups using GraphPad Prism software. The difference was considered statistically significant when a P value is less than 0.05.

**References**


