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## **Fibromodulin deficiency reduces LDL accumulation in atherosclerotic plaques in Apolipoprotein E-null mice**

Shami, Fibromodulin deficiency reduces LDL accumulation

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**Abstract:**

**Objective** The aim of this study was to analyze how an altered collagen structure affects development of atherosclerotic plaques.

**Methods and Results** Fibromodulin-null mice develop an abnormal collagen fibril structure. In apolipoproteinE- and apolipoproteinE/fibromodulin-null mice a shear stress-modifying carotid artery cast induced formation of atherosclerotic plaques of different phenotypes; inflammatory in low shear stress regions and fibrous in oscillatory shear stress regions. Electron microscopy showed that collagen fibrils were thicker and more heterogeneous in oscillatory shear stress lesions from apolipoprotein E/fibromodulin-null mice. Low shear stress lesions were smaller in apolipoprotein E/fibromodulin-null mice and contained less lipids. Total plaque burden in aortas stained en face with Oil red O, as well as lipid accumulation in aortic root lesions, was also decreased in apolipoproteinE/fibromodulin-null mice. In addition, lipid accumulation in RAW264.7 macrophages cultured on fibromodulin-deficient extracellular matrix was decreased while levels of interleukin-6 and -10 were increased. Our results show that an abnormal plaque collagen fibril structure can influence atherosclerotic plaque development.

**Conclusions** The present findings suggest a more complex role for collagen in plaque stability than previously anticipated in that it may promote lipid-accumulation and inflammation at the same time as it provides mechanical stability.

Keywords: Atherosclerosis, collagen, LDL, fibromodulin, carotid arteries.

Assembly of extracellular matrix (ECM) directs the conditions for both growth and stability of an atherosclerotic plaque. Vascular smooth muscle cells (SMCs) in the normal media are encased by a basement membrane embedded in collagens, adhesive proteins and proteoglycans.<sup>1</sup> At an early stage of the atherosclerotic process the SMCs in the media transfer from a contractile to a synthetic phenotype and migrate to the intima where they proliferate and produce a collagen-rich matrix.<sup>2</sup> During development of an atherosclerotic plaque there are marked changes in the structure of the ECM. About 60 % of the total protein content in the plaque represents fibril-forming collagens type I and III<sup>1,3</sup> and the roles of collagens in atherogenesis are multifaceted where excess collagen production can promote plaque growth and thereby contribute to vascular stenosis, whereas defective fiber assembly and/or degradation can cause plaque rupture and subsequent thrombosis.<sup>1</sup>

The collagen fibrillar structure is an important parameter in this aspect and it has been shown that SMCs seeded on monomeric type I collagen exhibit increased proliferation,<sup>4</sup> as well as migration,<sup>4,5,6</sup> when compared to cells plated on fibrillar type I collagen. An *in vivo* injury-experiment in pig coronary arteries supports this hypothesis by identifying a further connection between fibrillar collagen and inhibition of SMC proliferation through regulation of the cyclin-dependent kinase inhibitors p21<sup>Cip1</sup> and p27<sup>Kip1</sup>.<sup>7</sup>

Macrophage function also depends on the monomeric/polymeric state of type I collagen. Both spreading and MMP-9 production is inhibited in human macrophages when seeded on fibrillar type I collagen and increased when macrophages were seeded on monomeric type I collagen.<sup>8</sup> Furthermore, type I collagen can influence both macrophage phagocytic activity<sup>9</sup> as well as their uptake of LDL<sup>10</sup>. Therefore, it is clear that the physical state of type I collagen can have a great impact on both SMCs and macrophages and thus also on the developing atherosclerotic plaque.

Small leucine-rich repeat proteoglycans (SLRPs) are a group of structurally related proteins present in the ECM. Many of the SLRPs interact with collagens and regulate the assembly of fibrillar collagen matrices.<sup>11,12</sup> Fibromodulin is a 59 kDa SLRP primarily expressed in cartilage and tissues exposed to tensile stress, such as tendons<sup>13,14</sup>, and is a close homologue to lumican<sup>15</sup>. It regulates collagen fibril formation by binding collagen type I<sup>16</sup> and thereby influences the collagen scaffold formation<sup>17</sup> by a still unknown molecular mechanism. In fibromodulin-null mice abnormal collagen fibrils are found in tendons<sup>18,19</sup>, dermal wound healing<sup>20</sup>, liver cirrhosis<sup>21</sup> and tumor stroma of subcutaneous experimental tumors<sup>22</sup>. In the present study we tested the hypothesis that fibromodulin, by regulating the collagen structure, affects the development of atherosclerotic plaque tissue.

## Materials and Methods

Materials and Methods are available in detail in the online-only data supplement at <http://atvb.ahajournals.org>.

### Animals and *in vivo* alteration of shear stress

Animal tests were approved by the Malmö/Lund regional ethical committee (Sweden). ApoE-null mice in a B16 background and fibromodulin-null mice<sup>18</sup> were crossed and ApoE/fibromodulin-null mice were used in experiments with ApoE-null mice as controls. As described previously by Cheng et al<sup>23</sup>, standardized changes in shear stress were induced by a periaortic cast placed around the right carotid artery of ApoE- and ApoE/fibromodulin-null female mice kept on a cholesterol-rich diet, starting two weeks before surgery. Cast placement was performed on 18-week-old mice and mice were sacrificed at 30 weeks of age.

### Immunohistochemistry and histology

Carotid artery sections were stained using a Mac-2 antibody (Cedarlane; Burlington, Ontario, Canada), a smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA) antibody (Sigma-Aldrich), a fibromodulin antibody (gift from Prof. Dick Heinegård), the “PCNA staining kit” (Invitrogen) and a PCNA antibody (AbCam), and the TUNEL kit “TACS XLDAB In Situ Apoptosis Detection Kit” (Trevigen). Appropriate biotin-conjugated secondary antibodies were used and to differentiate staining patterns during sequential double staining 3,3'-Diaminobenzidine and streptavidin alkaline phosphatase with StayRed/AP (AbCam) were used with the different antibodies. For histology Oil Red O (0.3%, Sigma-Aldrich) and the “Accustain trichrome stain (Masson)” (Sigma-Aldrich) were used. All stained sections were visualized and digitalized using an Aperio ScanScope digital slide scanner (Scanscope Console v8.2.0.1263, AperioTechnologies, Inc., Vista, California, USA).

Lesion size is expressed as area and intima-media ratio and represents the mean value of four sections 15  $\mu$ m apart where the lesions were at their largest. Positively stained areas of Oil red O-stained descending aortas mounted en face were quantified and expressed as the percentage of the inner arterial lining covered by lesions. Aortic root sections were collected between first appearance and disappearance of the aortic valves and one section every 56  $\mu$ m was stained. Lipid content in plaques from the carotid artery and aortic root was expressed as the mean percentage of lesion area which stained positive for lipids.

### **Transmission electron microscopy**

Tissues were fixed in 0.15 M sodium cacodylate-buffered 2% glutaraldehyde, postfixed in 0.1 M-collidine-buffered 2% osmium tetroxide, and embedded in epoxy resin<sup>18</sup>.

### **RNA extraction and quantitative real time PCR (qRT-PCR)**

Due to small sample size, low and oscillatory shear stress plaques from three ApoE- and ApoE/fibromodulin-null mice were pooled, yielding two groups per genotype and uninjured carotid arteries from two mice were pooled, yielding three groups per genotype. Total RNA was extracted with RNeasy Micro Kit (Qiagen). Fifty ng total RNA was subjected to first strand cDNA synthesis with Superscript VILO cDNA Synthesis Kit (Invitrogen) and gene expression was analyzed using LightCycler software Version 3 (Roche Applied Science).

### ***In vitro* assays with RAW264.7 macrophages**

For ECM preparation aortic SMCs were isolated<sup>24</sup> from wild type and fibromodulin-null C57BL/6 mice and cultured cells were extracted with 0.5% Triton X-100 in PBS and 25 mmol/litre  $\text{NH}_4\text{OH}$  in PBS.<sup>25</sup> LDL-uptake was assessed by growing RAW264.7 cells on wild type and fibromodulin-null ECM with the addition of 50  $\mu$ l/ml native or  $\text{Cu}^{2+}$ -oxidized LDL in DMEM GlutaMax supplemented with 10% lipoprotein-deficient human serum, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco). After four days cells were fixed and stained with Oil Red O and the amount of lipids that had been taken up was quantified as Oil Red O-positive area relative to total cell area (values added from 20 micrographs per cover slip). Expression of fibromodulin by wild type SMCs was confirmed through Western blot (figure I in the online-only data supplement).

Cytokine concentrations in cell lysates were determined by using Mouse ProInflammatory 7-Plex Tissue Culture Kit (Meso Scale Discovery).

### **Software and statistical methods**

Sample size is expressed as n and error bars represent standard deviation (S.D.). Immunohistochemical and histological stains were quantified using BioPix iQ software (BioPixAB, Gothenburg Sweden). Two-tailed t-test (electron microscopy image analysis) was performed using Microsoft Excel. Mann-Whitney t-test (image analysis) and Student's t-test and

one-way ANOVA analysis with Bonferroni post-test (qRT-PCR) were performed using GraphPad Prism version 4.03 (GraphPad Software, San Diego California USA).

## Results

### **Fibromodulin deficiency reduces plaque size**

To test our hypothesis that an altered collagen structure affects the development of atherosclerotic plaque tissue we generated ApoE/fibromodulin-null double knock-out mice and studied the development of carotid atherosclerotic lesions of stable and vulnerable phenotypes induced by a perivascular cast.<sup>23</sup> Using this technique, vulnerable lesions develop under conditions of low shear stress proximal to the cast, while stable lesions develop distal to the cast due to oscillatory shear stress. Low shear stress plaques were smaller in ApoE/fibromodulin-null mice than in ApoE-null mice (figure 1A-B); the lesion/media ratio ( $p=0.005$ , figure 1E), total plaque area ( $p=0.006$ , figure 1F) and the length of the low shear stress plaques ( $484.4\pm 346.6$  vs.  $256.9\pm 232.3$ ,  $p=0.013$ ) were reduced in the ApoE/fibromodulin-null lesions. Note that there was no difference in the size of the oscillatory shear stress lesions between the two genotypes (figure 1C-D).

Low shear stress carotid lesions contained less collagen than the oscillatory shear stress lesions in both ApoE-deficient ( $p=0.0011$ ) and ApoE/Fmod-null mice ( $p=0.0010$ ), but there was no difference in collagen (figure II in the online-only data supplement), SMC or T cell content (data not shown) between the genotypes. Additionally, we found no difference in the size of aortic root lesions (figure III in the online-only data supplement).

### **Increased collagen fibril thickness in fibromodulin-deficient low shear stress plaques**

Fibromodulin expression has previously been demonstrated in murine aortic atherosclerotic lesions.<sup>26</sup> In addition, we found positive fibromodulin immunoreactivity in carotid lesions and, to some extent, in the medial layer associated with the lesions (figure IVA and C in the online-only data supplement). No positive fibromodulin staining was found in the ApoE/fibromodulin-null lesions or in uninjured control arteries (figure IVB, D-F in the online-only data supplement). These results are in agreement with the presence of fibromodulin transcripts in carotid lesions in ApoE-null mice, whereas no transcripts could be detected in ApoE/fibromodulin-null carotid lesions (figure IVG in the online-only data supplement).

Electron microscopy of atherosclerotic oscillatory shear stress lesions from ApoE/fibromodulin-null mice revealed an altered structure of the collagenous matrix manifested by the presence of more heterogeneous collagen fibrils (figure 2A and B). The average fibril diameter thickness was increased from 25 nm to 34 nm ( $P<0.0001$ ; figure 3C) in ApoE/fibromodulin-null compared with ApoE-null lesions. Collagen fibrils in low shear stress lesions were disorganized to an extent that they were not quantifiable, however, the surrounding adventitia contained proper collagen fibrils (Shami, unpublished data, 2011). We found no difference between collagen fibril diameters in healthy, uninjured carotid arteries or in descending aortas from ApoE- and ApoE/fibromodulin-null mice (figure V in the online-only data supplement).

### **Fibromodulin deficiency reduces lipid accumulation in plaques**

Lipid retention is an essential process in atherosclerosis development<sup>2</sup> and ECM components are known to affect LDL-retention.<sup>25</sup> We analyzed the lipid content of low and oscillatory shear stress plaques in ApoE- and ApoE/fibromodulin-null by Oil Red O-staining. Reduced lipid accumulation was observed in low (figure 3A), but not in oscillatory (figure VIB in the online-only data supplement), shear stress carotid plaques from ApoE/fibromodulin-null mice ( $p=0.028$ ).

A similar decrease in lipid accumulation was observed in the media surrounding these plaques ( $p=0.0019$ , figure VIA in the online-only data supplement). We also analyzed lipid accumulation in aortic root lesions that were stained with Oil Red O and found that the lipid content was lower in lesions from ApoE/fibromodulin-null mice ( $p=0.0056$ , figure 3B). In an atherosclerotic plaque lipids primarily accumulate within macrophages and foam cells.<sup>27</sup> Consequently, when comparing macrophage accumulation in lesions from ApoE- and ApoE/fibromodulin-null mice we also found a significant decrease in total macrophage content in low shear stress ApoE/fibromodulin-null plaques ( $p=0.0214$ , figure 3C-G).

In addition, to compare the general severity of atherosclerosis between mice with or without fibromodulin-expression, we performed en face Oil Red O-staining of the descending aorta and found a 30% decrease ( $p=0.0144$ ) in total plaque burden in aortic lesions from ApoE/fibromodulin-null mice (figure 4).

### **A fibromodulin-deficient ECM reduces lipoprotein uptake by macrophages *in vitro***

To further explore the mechanisms involved in the reduced lipid accumulation in low shear stress lesions from fibromodulin-deficient mice we analyzed lipid uptake in macrophages cultured on fibromodulin-null ECM synthesized by SMCs. RAW264.7 macrophages were seeded on wild type or fibromodulin-null ECM and native or oxidized LDL was added to the culture medium. After four days cells had grown to confluence (approximately 6350 cells/mm<sup>2</sup>) and were stained with Oil Red O. Our results demonstrate a decreased accumulation of oxidized LDL in cells growing on a fibromodulin-deficient ECM compared to cells growing on a wild type ECM ( $p=0.018$ , figure 5A). There was no difference in the uptake of native LDL between RAW264.7 macrophages cultured on wild type or fibromodulin-deficient ECM.

We also analyzed the migratory capacity of RAW264.7 macrophages and found no difference in migration between cells seeded on wild type and fibromodulin-deficient collagen (data not shown).

### **A fibromodulin-deficient ECM alters cytokine production *in vitro***

Cytokines and chemokines produced by leukocytes in plaques play an important part in atherosclerosis development<sup>28</sup> including regulation of uptake and efflux of cholesterol<sup>29</sup>. Accordingly, we analyzed if an altered ECM also can have an impact on macrophage cytokine production. RAW264.7 cells were cultured on wild type and fibromodulin-deficient ECM for two days in presence of native or oxidized LDL. The levels of interleukin-10 (IL-10) and interleukin-6 (IL-6) were found to be increased in cells cultured on fibromodulin-deficient ECM in presence of oxidized LDL ( $p=0.0028$  and  $0.04$ , respectively, figure 5B and C). However, we found no differences in levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 (IL-1) or interleukin-12 (IL-12) between cells growing on wild type and fibromodulin-deficient ECM (data not shown).

In addition, we found no difference in plasma levels of inflammatory markers, including tumor necrosis factor- $\alpha$ , interferon- $\gamma$ , interleukin-1 $\beta$ , -2, -4, -5, -10, -12 or keratinocyte-derived chemokine (KC) at the time animals were sacrificed (figure VII in the online-only data supplement). There was no difference in plasma cholesterol levels (figure VIII in the online-only data supplement).

### **Fibromodulin deficiency increases cell proliferation and collagen turnover**

Proliferation of SMCs is another important mechanism regulating atherosclerotic plaque growth. The fibrillar state of collagen matrix can influence this process; the monomeric or degraded form stimulates and the mature fibrillar state can inhibit cell proliferation.<sup>4</sup> Thus, it is likely that the abnormal fibril formation in ApoE/fibromodulin-null mice affects cell proliferation in the atherosclerotic plaque. Low and oscillatory shear stress carotid lesions isolated from ApoE- and ApoE/fibromodulin-null mice were stained for proliferating cell nuclear antigen (PCNA) and the

proportions of positively stained nuclei, i.e. proliferating cells, was determined (figure 6A-E). In ApoE/fibromodulin-null mice, proliferation was increased by 102% in low shear stress lesions ( $p=0.0014$ ) and by 110% in oscillatory shear stress lesions ( $p=0.0032$ ).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) together with quantification of plaque cell numbers showed that the increased cell proliferation in lesions from mice lacking fibromodulin was not accompanied by a parallel increase in apoptosis (figure 6F and figure IX in the online-only data supplement), but rather by an overall increase in cell density in both low and oscillatory shear stress plaques ( $p=0.0012$  and  $0.0108$ , respectively, figure 6G).

Double staining that combined the proliferation marker PCNA and the macrophage marker Mac2 or SMC marker  $\alpha$ -SMA showed that the proliferating cells consisted of both SMCs and macrophages. The latter were mainly found in lesions from the low shear stress region in both ApoE- and ApoE/fibromodulin-null mice (figure X in the online-only data supplement).

As cell proliferation is closely related to tissue turnover, we analyzed the expression of procollagen as well as enzymes participating in procollagen processing. Quantitative RT-PCR demonstrated a significant increase in the expression of procollagen chain  $1\alpha 2$  (Coll1 $\alpha 2$ ) in low shear stress lesions from ApoE/fibromodulin-null compared to ApoE-null mice (figure XI, in the online-only data supplement). This increase was significant also in comparison to oscillatory shear stress plaques as well as to control arteries within the same genotype. A similar tendency was found in the expression of procollagen chain  $1\alpha 1$  (Coll1 $\alpha 1$ ) (figure XI in the online-only data supplement).

The mRNA expression of lysyl oxidase (Lox), the main collagen cross-linking enzyme<sup>30</sup>, was also enhanced in low shear stress lesions from ApoE/fibromodulin-null mice (figure XI in the online-only data supplement). In addition, the basal expression level of Lox was significantly higher in control arteries derived from ApoE/fibromodulin-null mice when compared to ApoE-null mice, indicating a general increase in collagen turnover in mice lacking fibromodulin expression.

As tissue turnover also includes degradation we analyzed the expression of urokinase plasminogen activator receptor-associated protein (uParap), an internalizing receptor that binds degraded collagen fragments for further uptake and degradation<sup>31</sup>. uParap expression was significantly increased in oscillatory shear stress plaques from ApoE/fibromodulin-null mice compared with ApoE-null mice (figure XI in the online-only data supplement) and showed a tendency of higher expression in low shear stress plaques. uParap expression was also significantly increased in low shear stress lesions compared to oscillatory shear stress lesions in both genotypes.

## Discussion

The aim of the present study was to analyze how an altered collagen structure affects the development of atherosclerotic plaques in ApoE-null mice. Fibromodulin is a collagen-binding protein with an important role in collagen fiber assembly.<sup>18, 32</sup> We crossed fibromodulin- and ApoE-null mice to generate atherosclerotic plaques with a defective collagen structure. In line with the previously published data on collagen-structure in fibromodulin-null tendons<sup>18</sup>, cornea<sup>33</sup>, cartilage<sup>34</sup> and wound healing<sup>20, 35</sup>, the ApoE/fibromodulin-null mice developed atherosclerotic lesions with more heterogeneous and significantly larger fibrils resulting in a disorganized collagenous matrix. However, lack of fibromodulin did not alter the collagen content in the lesions.

However, we found plaque size, lipid accumulation and macrophage content to be significantly decreased in lesions from ApoE/fibromodulin-null mice. It is possible that the decreased lipid content reduces the local inflammatory response and thus also affects the size of



the plaque. Our finding that the total amount of macrophages is decreased is in favor of this hypothesis. Furthermore, collagen type I fibrils appear to be involved in lipid-retention and macrophage accumulation. *In vitro* experiments have previously shown that collagen type I stimulated monocyte differentiation as well as uptake of lipids by macrophages.<sup>10</sup> Thus, the abnormal collagen fibrils in ApoE/fibromodulin-null lesions may have reduced ability to stimulate lipid accumulation in macrophages, resulting in reduced lipid content in these lesions. Indeed, our finding that RAW264.7 macrophages cultured on fibromodulin-null ECM has a decreased capacity to accumulate oxLDL supports this hypothesis.

Furthermore, the decrease in lipid accumulation in RAW264.7 macrophages grown on fibromodulin-deficient ECM was associated with an increased production of IL-6 and -10. In line with this, Frisdal et al<sup>29</sup> reported that treatment with IL-6 promoted an anti-inflammatory cytokine profile, including increased secretion of the anti-atherogenic cytokine<sup>36,37</sup> IL-10, as well as efflux of excess cholesterol from macrophages *in vitro* through ABCA1 (ATP binding cassette A1).

Collagen structure has general effects on cellular functions, but may also specifically affect the binding of LDL or oxidized LDL to the ECM. The tyrosine sulfate-rich N-terminal domain of fibromodulin has heparin-like properties, binds basic heparin-binding proteins and may also bind LDL<sup>38</sup>. Kaplan and Aviram<sup>39</sup> showed that the larger ECM GAG-content in old mice resulted in a greater binding and uptake of oxidized LDL compared to younger mice, and Tran-Lundmark et al.<sup>25</sup> performed a study where LDL in an ApoE-null mouse with ECM containing heparan sulfate-deficient perlecan was shown to diffuse more rapidly into the vessel wall, but exhibited a reduced retention rate. Similarly, ECM synthesized in the absence of fibromodulin may be altered in a manner where LDL-retention is reduced.

Although fibromodulin deficiency reduces lipid content in plaques both at the aortic root and at the low shear stress region of the cast, it only attenuates the size of the latter type of lesion. The reason for this discrepancy remains to be fully elucidated, but may involve differences in shear stress patterns; the shear stress on the vessel wall is different in the aortic root compared to the low shear stress region proximally to the cast in the carotid artery. Van Doormaal et al.<sup>40</sup> demonstrated complex flows and a high oscillatory shear stress in the aortic root while an oscillatory shear stress to the vessel wall is induced in the oscillatory shear stress region distally, but not proximally, to the cast in our model.<sup>23</sup> Interestingly, in similarity to the aortic root lesions, there was no difference in the size of the distal lesions when comparing the two genotypes. This should be compared to the low shear stress on the vessel wall in the proximal region of the cast where we could detect differences in lesion size.

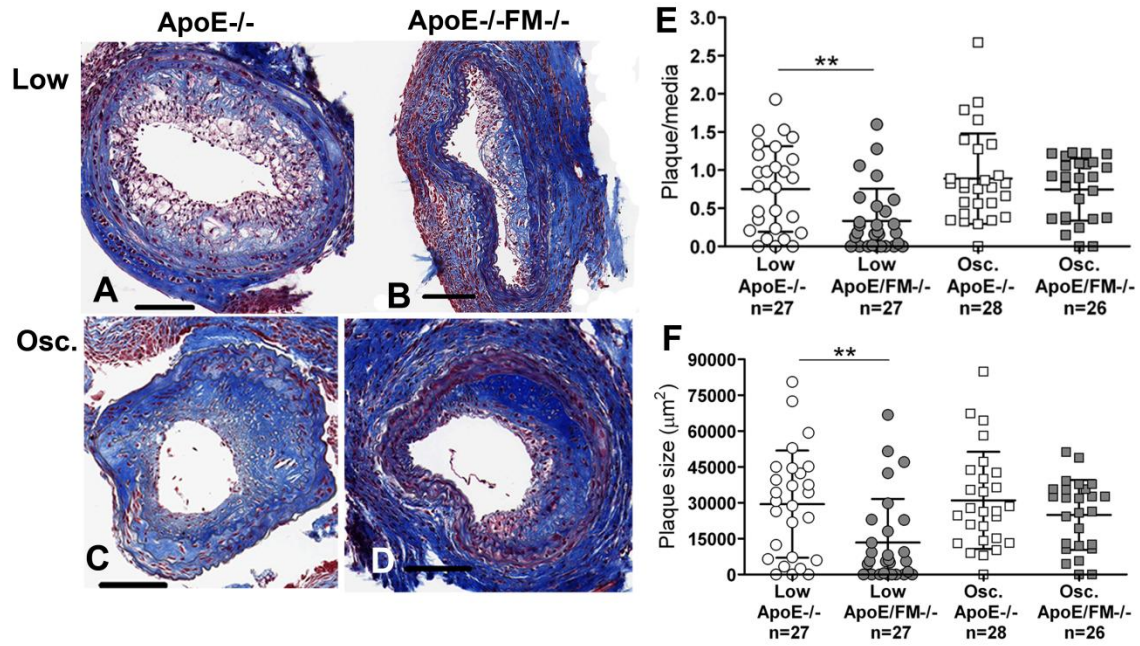
In a study by Ranjzad et al.<sup>41</sup> both the neointima and the neointimal collagen content was found to be decreased in human veins after overexpression of fibromodulin through adenovirus-mediated gene transfer *ex vivo*. The effects were found to be mediated, at least in part, by antagonism of TGF- $\beta$ 1 and collagen homeostasis. There are several possible explanations to these apparently conflicting results. The present study focused on atherosclerotic plaque development while the study by Ranjzad investigated neointimal hyperplasia; two processes that develops through different mechanisms. Thus, in our study the effects of fibromodulin deficiency on plaque size are found to be principally due to alterations in lipid accumulation and macrophage lipid uptake while, in contrast, overexpression of fibromodulin during neointimal hyperplasia were found to affect SMC and collagen accumulation. In addition, the discrepancies may be explained by differences in the experimental models used. The present study was performed on atherosclerotic lesions developed over 20 weeks in ApoE and the ApoE/fibromodulin deficient mice, whereas the study of Ranjzad et al was performed utilizing an *ex vivo* model with human veins maintained for 14 days in culture. However, it is interesting to note that results on cell density from the two studies are in agreement as Ranjzad et al found neointimal cell density to be lowered after fibromodulin overexpression, while in the present study cell density in atherosclerotic lesions was increased by fibromodulin deficiency.

We found that cell proliferation was increased in both low and oscillatory shear stress lesions in mice lacking fibromodulin. As there is no correlation between fibromodulin expression and proliferation rate in individual plaques (Shami, unpublished data, 2011), we speculate that the increased cell proliferation and decreased lipid retention is instead directly affected by an altered surrounding collagen structure and that the increase in cell proliferation reflect an increased matrix turnover. To test this hypothesis we quantified RNA transcripts for procollagens  $\text{Coll}\alpha 1$  and  $\text{Coll}\alpha 2$  and the collagen-turnover related *Lox* and *uParap* in the plaques and in control arteries. The results indicated a higher collagen turnover in carotid lesions from ApoE/fibromodulin-null mice compared to ApoE-null mice. An increased collagen turnover resulting in a higher proportion of degraded or monomeric, non-fibrillar collagen may further enhance the disordered collagen scaffold in plaques lacking fibromodulin expression. This milieu could in turn stress the cells in the plaque to an increased rate of proliferation. In support of this conclusion, Koyama et al. demonstrated that intact fibrillar collagen has an inhibitory effect on proliferation while degraded or monomeric collagen promotes proliferation.<sup>4</sup> Collagen type VIII, produced and deposited by SMCs, is suggested to cover native collagen type I and thereby allow cells to overcome the inhibition of proliferation<sup>42</sup>; a process that cell proliferation in fibromodulin-null lesions may not be dependent on. We speculate that abnormal collagen type I-fibrils in fibromodulin-deficient ECM thus may lead to decreased inhibitory effects as well as increased stimulatory effects on proliferation compared to normal collagen fibrils of a fibromodulin-competent matrix. The increase in proliferation rate in cells embedded in a fibromodulin-deficient matrix may also be explained by altered downstream signaling caused by a different expression of cell surface collagen receptors, such as integrins and discoidin domain receptors (DDR).

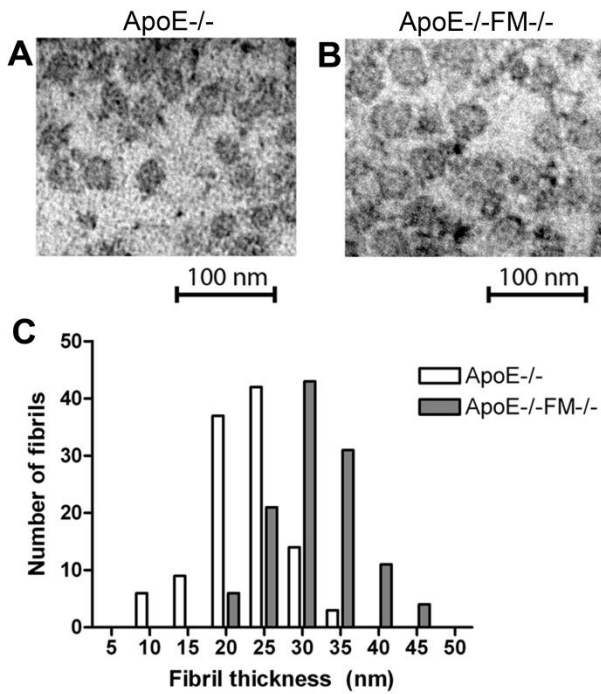
It is likely that SMCs can adjust to an altered collagen matrix already during early vessel development. This adjustment can include different expression patterns of cell surface receptors, such as DDRs (collagen-activated tyrosine kinases)<sup>43</sup>, and subsequently affect the response, and thus behavior, of the cells. Hou et al. has shown that deletion of DDR-1 attenuates plaque development<sup>44</sup> and shifts plaque composition toward reduced inflammation as well as an early increase in ECM content and SMC proliferation.<sup>45</sup>

In this study we used low and oscillatory shear stress to induce the formation of vulnerable and stable atherosclerotic lesions, respectively, in ApoE-null mice. We demonstrate that a defective formation of collagen fibers due to fibromodulin-deficiency reduces the formation of lipid-rich vulnerable lesions but does not affect the formation of more stable lesions induced by oscillatory shear stress. The present findings suggest a more complex role for collagen in plaque stability than previously anticipated in that it may promote lipid-accumulation and inflammation at the same time as it provides mechanical stability.

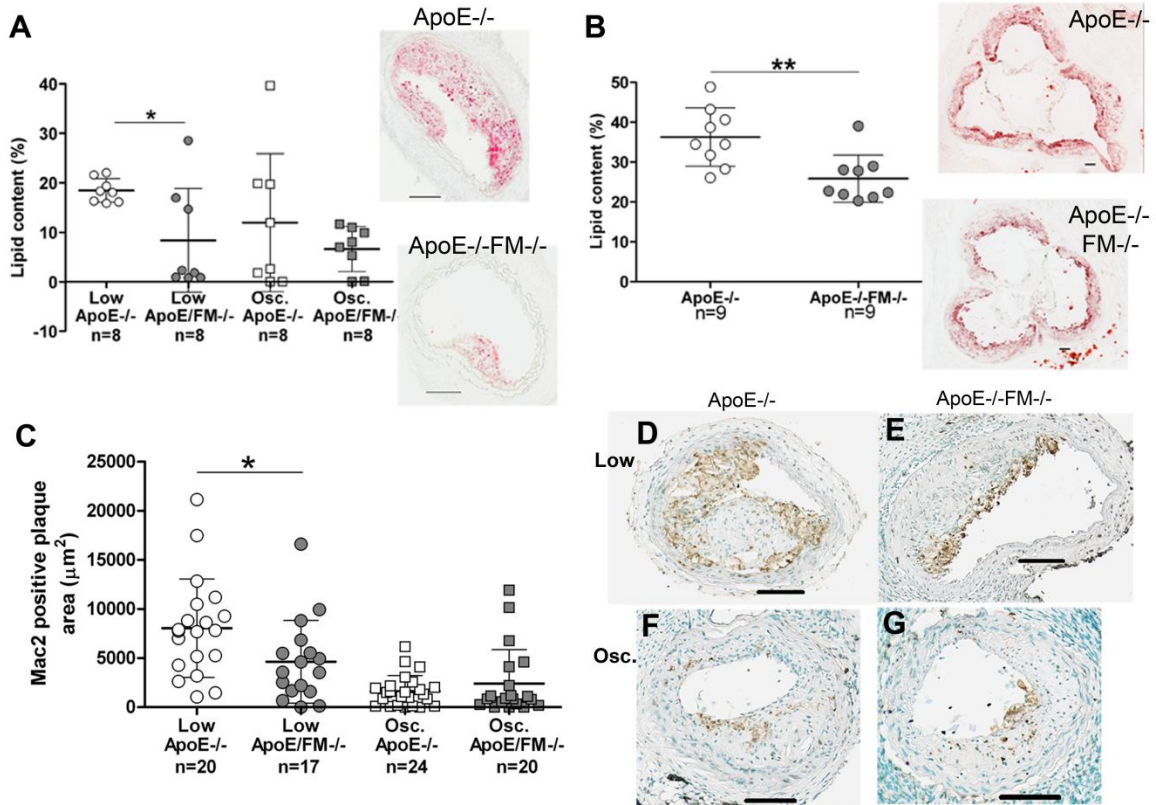
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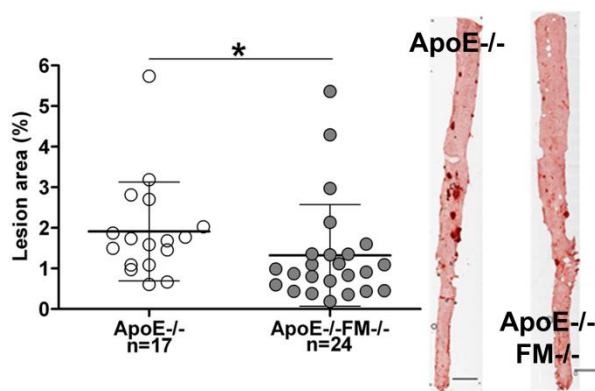
**Figure 1: Decreased size of low shear stress lesions in ApoE/fibromodulin-null (ApoE<sup>-/-</sup>-FM<sup>-/-</sup>) mice.** Representative sections of Masson's trichrome-stained carotid lesions (A-B; low shear stress, C-D; oscillatory (Osc.) shear stress). Intima/media ratio is shown in (E) and total plaque area in (F). Scale bars = 100 μm.



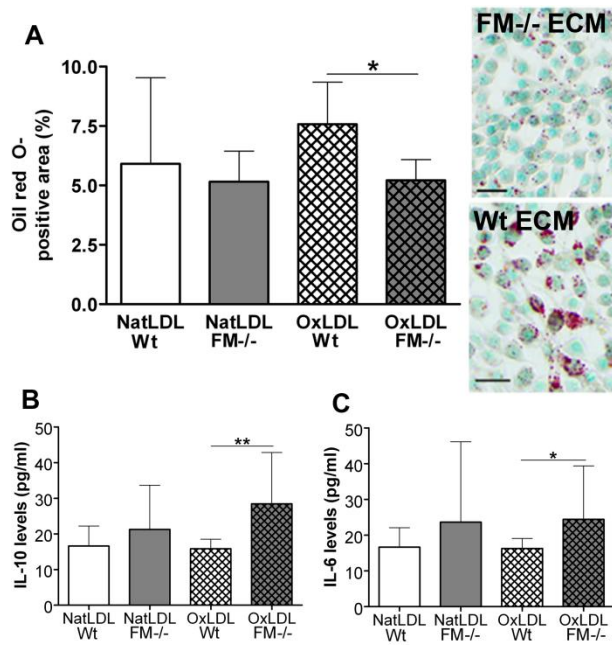
**Figure 2: Thicker and more heterogeneous collagen fibrils in ApoE/fibromodulin-null lesions.** Electron micrographs of atherosclerotic carotid lesions (n=3) of ApoE- (A) and ApoE/fibromodulin-null mice (B). Image analysis shown in (C).



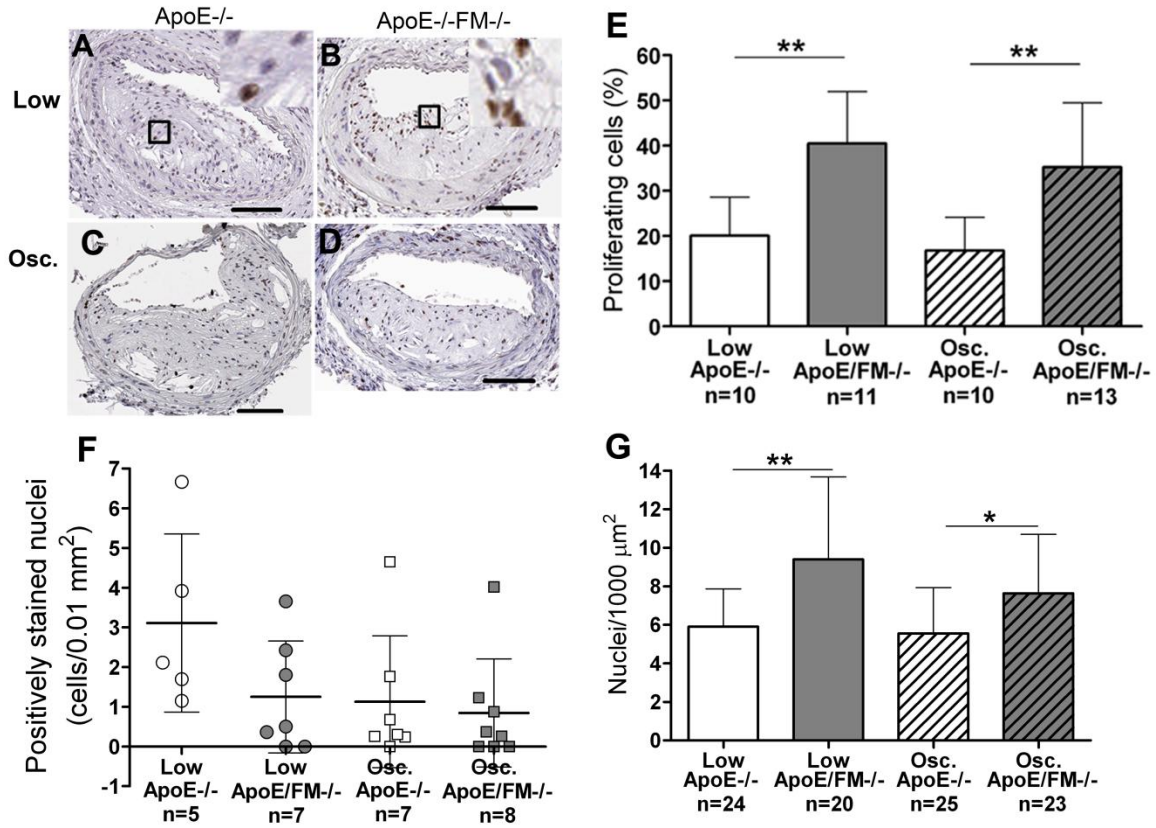
**Figure 3: Decreased lipid and macrophage content in low shear stress lesions in ApoE/fibromodulin-null mice.** Image analysis of Oil red O-stained low and oscillatory shear stress carotid plaques (A; representative low shear stress lesions shown) and sections from aortic root lesions (B). Image analysis of mac2-stained low shear stress and oscillatory shear stress ApoE- and ApoE/fibromodulin-null carotid plaques (C-G). Scale bars = 100 µm.



**Figure 4: Decreased plaque burden in ApoE/fibromodulin-null mice.** Flat preparations of descending aortas from ApoE- and ApoE/fibromodulin-null mice. Scale bars = 1 mm.



**Figure 5: Altered macrophage lipid uptake and cytokine production *in vitro*.** Uptake of native and oxidized LDL by RAW264.7 cells cultured on wild type and fibromodulin-deficient ECM (A, scale bars = 20µm). Levels of IL-10 (B) and Il-6 (C) in cell lysates from RAW264.7 cells cultured on wild type and fibromodulin-deficient ECM, stimulated by native or oxidative LDL; and PMA/ionomycin.



**Figure 6: Increased cell proliferation in carotid lesions.** Representative PCNA-stained sections of lesions from ApoE<sup>-/-</sup> (A and C) and ApoE/fibromodulin-null (B and D) mice; black squares represent insets. Image analysis of the percentage of PCNA-positive cells in low and oscillatory shear stress lesions from ApoE<sup>-/-</sup> and ApoE/fibromodulin-null mice (E). TUNEL stain (F) and cell density (G) in low and oscillatory shear stress plaques from ApoE<sup>-/-</sup> and ApoE/fibromodulin-null mice. Scale bars = 100 μm.

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## Disclosures

None.

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