**Athsq1** Is an Atherosclerosis Modifier Locus With Dramatic Effects on Lesion Area and Prominent Accumulation of Versican

Sara Bretschger Seidelmann, Chaoiling Kuo, Nick Pleskac, Jennifer Molina, Scott Sayers, Rong Li, Jing Zhou, Pamela Johnson, Kathleen Braun, Christina Chan, Daniel Teupser, Jan L. Breslow, Thomas N. Wight, Alan R. Tall, Carrie L. Welch

**Objective**—Susceptibility to atherosclerosis is genetically complex, and modifier genes that do not operate via traditional risk factors are largely unknown. A mouse genetics approach can simplify the genetic analysis and provide tools for mechanistic studies.

**Methods and Results**—We previously identified atherosclerosis susceptibility QTL (Athsq1) on chromosome 4 acting independently of systemic risk factors. We now report confirmation of this locus in congenic strains carrying the MOLF-derived susceptibility allele in the C57BL/6J-Ldlr–/– genetic background. Homozygous congenic mice exhibited up to 4.5-fold greater lesion area compared to noncongenic littermates (P<0.0001). Analysis of extracellular matrix composition revealed prominent accumulation of versican, a presumed proatherogenic matrix component abundant in human lesions but almost absent in the widely-used C57BL/6 murine atherosclerosis model. The results of a bone marrow transplantation experiment suggested that both accelerated lesion development and versican accumulation are mediated, at least in part, by macrophages. Interestingly, comparative mapping revealed that the Athsq1 congenic interval contains the mouse region homologous to a widely-replicated CHD locus on human chromosome 9p21.

**Conclusion**—These studies confirm the proatherogenic activity of a novel gene(s) in the MOLF-derived Athsq1 locus and provide in vivo evidence for a causative role of versican in lesion development. *(Arterioscler Thromb Vasc Biol. 2008;28:2180-2186.)*

**Key Words:** atherosclerosis • congenic strain • genetics • extracellular matrix • mapping

Susceptibility to atherosclerosis is influenced by both genetic and environmental factors, with approximately 40% to 60% of interindividual variation attributed to genetic factors.¹ The complex etiology has hampered genetic studies of atherosclerosis in humans per se until recently. Early studies used the candidate gene approach to identify rare genetic variants contributing to traditional risk factors including plasma levels of LDL/VLDL, HDL, lipoprotein (a), homocysteine, and blood pressure.²⁻⁶ Now many of the genes underlying Mendelian forms of dyslipidemia have been shown to contribute to population variation of plasma lipids using genome-wide association (GWA) studies.⁷⁻⁹ Attempts to identify genes directly underlying coronary artery disease (CAD)/myocardial infarct (MI) were first pursued through linkage studies of families enriched for disease¹⁰⁻¹⁴ and large-scale association studies.¹⁵⁻¹⁸ However, the first locus to be widely-replicated for CHD was recently revealed through a series of GWA studies.¹⁹⁻²² A common variant was localized to chromosome (chr) 9p21, but the underlying gene and mechanism of action are unknown. Thus, while a number of genes contributing to traditional risk factors have been identified, few of the genes underlying nontraditional risk factors are known.

Animal models offer an alternative approach for the genetic analysis of complex diseases such as atherosclerosis. In early work, the existence of atherosclerosis susceptibility loci in the mouse model was suggested using recombinant inbred strains of mice.²³⁻²⁶ Recently, the chromosomal locations of such loci have been mapped using linkage analysis of experimental crosses.²⁷⁻³¹ We previously reported the localization of atherosclerosis susceptibility QTL 1 (Athsq1) to chr 4 using a cross between strains MOLF/Ei and C57BL/6J-Ldlr⁻⁻.²⁸ The effect of Athsq1 was independent of systemic risk factors, suggesting a local effect within the vessel wall. We now report confirmation of Athsq1 in congenic strains carrying a 51-megabase (Mb) interval (648 genes). Lesions...
derived from the congenic mice exhibited prominent accumulation of versican, a major extracellular matrix (ECM) proteoglycan component of human lesions not previously observed in mouse lesions. We also show that both the accelerated atherogenesis and versican accumulation are mediated, at least in part, by bone marrow (BM)-derived cells.

**Methods**

An expanded Methods section is available in the Supplemental Data (available online at http://atvb.ahajournals.org).

**Mice**

MOLF/Ei (MOLF) and B6.129S7-Ldlr<sup>−/−</sup> (B6-Ldlr<sup>−/−</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, Me). B6.MOLF-Atthsq1 congenic mice were generated by introgression of the MOLF donor interval into the B6-Ldlr<sup>−/−</sup> background using a marker-assisted method. Mice designated m<sup>o</sup>/m<sup>o</sup> represent a subcongenic strain derived from the m/m strain. Mice (males and females) were fed Western-type diet (WTD) for 12- or 6-weeks before sacrifice as indicated. All procedures were in accordance with institutional guidelines.

**DNA Extraction and Ldlr Genotyping**

DNA was extracted from tail tips and microsatellite markers and Ldlr alleles were typed by polymerase chain reaction (PCR) as previously described.

**Atherosclerotic Lesion Measurements**

Serial sections were prepared from the aortic root as described. Lesion area was quantified by video microscopy and average lesion size determined from 5 sections per mouse. Necrotic core area was determined from H&E-stained sections as the acellular area beneath the fibrous cap. Cap thickness was determined from Verhoeff-stained sections using a scoring system based on numbers of elastic layers as described. Collagen was detected with Masson’s trichrome stain (Poly Scientific).

**Immunohistochemistry**

Versican was detected in aortic sections using a rabbit polyclonal antibody against the β-gag domain (Chemicon International).

**Protein and mRNA Quantification of Versican**

Proximal aortas were collected from 6-week WTD-fed mice. Proteoglycans were extracted, isolated by chromatography, and digested with chondroitin ABC lyase as described. Immunoblots were probed with mouse β-gag antibody. Relative abundance of versican was determined by quantitative image analysis. cDNA synthesis from total RNA was carried out using standard procedures. Real-time PCR was performed using Taqman Gene Expression Assays (available online at http://atvb.ahajournals.org).

**BM Transplantation**

BM transplantation was performed as described. Irradiated B6-Ldlr<sup>−/−</sup> mice (all females) were injected with BM derived from either m<sup>o</sup>/m<sup>o</sup> congenic or b/b noncongenic mice. Reconstitution of the BM with donor cells was checked at 6 weeks postinjection using microsatellite marker D4Mit185. Mice were then fed WTD for 11 weeks, euthanized, and the aortic roots dissected and sectioned as above.

**Statistical Analysis**

ANOVA and t tests were performed with or without square root transformation, as indicated, using STATVIEW 5.0 (Abacus Concepts Inc). Data are mean±SD. The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

**Results**

**B6.MOLF-Atthsq1 Congenic Mice Exhibit Accelerated Lesion Development**

To confirm the Atthsq1 susceptibility locus and test its amenability to fine mapping, we created congenic mice carrying one or two copies of the MOLF-derived susceptibility allele from chr 4 on a B6-Ldlr<sup>−/−</sup> background. The full congenic interval extended from D4Mit185 (43 cM) to D4Mit42 (81 cM) (Figure 1A). However, homozygous mice carrying the full interval exhibited a high rate of mortality shortly after weaning. The cause of death was unlikely attributable to atherosclerotic complications because the mice died before the start of WTD feeding and, thus, before the onset of atherosclerotic disease. Subcongenic mice carrying a shorter interval, extending from D4Mit185 (43 cM) to D4Mit70 (62 cM) (Figure 1A), developed normally in the homozygous state. After 12 weeks of WTD feeding, mean atherosclerotic lesion area was significantly greater in Atthsq1 heterozygotes (b/m) and Atthsq1 homozygotes carrying the full interval (m/m) or the subcongenic interval (m<sup>o</sup>/m<sup>o</sup>) compared to noncongenic littermates (b/b) (Figure 1B). The differences in lesion area were independent of plasma cholesterol levels, glucose, body weight, or sex (supplemental Table I and supplemental Figure I). Homozygous mice were maintained as m<sup>o</sup>/m<sup>o</sup> (carrying the shorter interval) for the remaining experiments.

Although the congenic strains exhibited greater lesion area compared to noncongenics after 12-week WTD feeding, lesion development was extensive in all groups. We then examined lesions at an earlier time point and found the effect of Atthsq1 to be more dramatic. After 6-week WTD feeding, b/m heterozygotes exhibited almost 2-fold and m<sup>o</sup>/m<sup>o</sup> homozygotes exhibited 4.5-fold greater lesion area compared to noncongenic b/b controls. The lesion area values at the 6-week time point were 93 176±35 385 μm<sup>2</sup>/section (m<sup>o</sup>) versus controls (b/m; 220 577±153 238 μm<sup>2</sup>/section (m<sup>o</sup>/m<sup>o</sup>), and 52 526±17 512 μm<sup>2</sup>/section (b/b) (P<0.05 b/m versus b/b; and P<0.0001, m<sup>o</sup>/m<sup>o</sup> versus b/b) (Figure 1B).

Analysis of lesion composition revealed dramatic differences between congenic and noncongenic mice at the 6-week time point. While noncongenic littermates exhibited small, focal, fatty streak lesions (Figure 2A, 2D, 2G, and 2J), Atthsq1 b/m heterozygotes developed both fatty streak lesions and more advanced fatty-fibrous lesions characterized by fibrous cap and necrotic core formation (Figure 2B, 2E, 2H, and 2K). In stark contrast to Atthsq1 b/b controls, Atthsq1 m<sup>o</sup>/m<sup>o</sup> homozygotes exhibited advanced fibrous lesions, often covering the entire circumference of the vessel wall (Figure 2C, 2F, 2I, and 2L). The most advanced lesions from control mice exhibited thin (single elastic layer) fibrous caps, whereas advanced lesions from b/m and m<sup>o</sup>/m<sup>o</sup> congenic mice exhibited intermediate (two to four elastic layers) or thick (greater than four elastic layers) fibrous caps (P<0.0002 for homozygotes versus controls). Mean necrotic core area, defined as the acellular area encapsulated by the cellular regions of the cap and shoulders, was significantly greater in congenic mice (10 000±4000 μm<sup>2</sup>/section [b/m] and 32 000±11 000 μm<sup>2</sup>/section [m<sup>o</sup>/m<sup>o</sup>]) compared to noncongenic controls.
Thus, the congenic mice exhibited greatly accelerated atherosclerotic lesion development in a gene dosage-dependent manner.

**Atherosclerotic Lesions of Atshq1 Congenic Mice Exhibit Prominent Accumulation of the Proatherogenic Matrix Component, Versican**

Atherogenesis is initiated by lipoprotein retention and modification, ECM deposition, and inflammatory cell recruitment. To gain insight into the mechanism of accelerated atherogenesis in Atshq1 congenic mice, we examined ECM composition of atherosclerotic lesions using a variety of special stains and antibodies. Staining for collagen, elastin, and hyaluronan showed no obvious differences in comparable lesions derived from Atshq1 m*/m* congenic and Atshq1 b/b noncongenic mice (data not shown). However, immunostaining for versican revealed dramatic accumulations in lesions derived from congenics but not controls. Whereas versican staining was almost undetectable in nonlesional aorta of both control and congenic mice (3.5 weeks WTD, data not shown), staining was prominent in the medial area beneath plaques and was also observed in cellular regions of the intima (Figure 3A through 3D). In 6-week WTD-fed mice, the mean versican-positive medial area was more than 10-fold greater in Atshq1 m*/m* congenic and Atshq1 b/b noncongenic mice (data not shown). However, immunostaining for versican revealed dramatic accumulations in lesions derived from congenics but not controls. Whereas versican staining was almost undetectable in nonlesional aorta of both control and congenic mice (3.5 weeks WTD, data not shown), staining was prominent in the medial area beneath plaques and was also observed in cellular regions of the intima (Figure 3A through 3D). In 6-week WTD-fed mice, the mean versican-positive medial area was more than 10-fold greater in Atshq1 m*/m* congenic strains compared to Atshq1 b/b controls after 6-week WTD feeding (Figure 4A and 4B). By quantitative PCR, we observed no difference in versican mRNA levels between the strains (Figure 4C). Furthermore, we observed no differences in mRNA levels of hyaluronase-1 and -2, CD44, or hyaluronan synthase-1 and -2, but a modest decrease in hyaluronan synthase-3 in the congenics (supplemental Figures III and IV). These data suggest a posttranscriptional mechanism regulating accumulation of versican.

**Accelerated Atherosclerosis and Versican Accumulation in Atshq1 Congenic Mice Is Mediated, at Least in Part, by BM-Derived Cells**

Because the effect of Atshq1 was more dramatic at the earlier time point, and these lesions are enriched with macrophages (compared to smooth muscle cells by immunostaining) (supplemental Figure V), we performed a BM transplantation experiment to test the role of macrophages in the congenic model. Lethally-irradiated B6-Ldlr−/− recipient mice were injected with donor BM derived from congenic or noncongenic mice (both on the B6-Ldlr−/− background). The mice were fed WTD for 11 weeks after recovery/repopulation of the BM-derived cells with donor cells. Lesion area was increased (300±1200 μm²/section; P<0.04 and P<0.0006, respectively).

![Figure 1.](attachment://image_url)
significantly increased in mice receiving congenic (m*/m*)-derived BM compared to mice receiving noncongenic (b/b) BM ($P=0.0016$; Figure 5A). The fold-difference in lesion area between mice receiving congenic or control BM (1.6-fold) was similar to that shown in Figure 1B, left panel (1.8-fold) after feeding the WTD for similar time periods (11 or 12 weeks). There was also greater accumulation of versican in mice receiving congenic-derived BM (Figure 5B and 5C): a 2.5-fold increase in versican-positive medial area (Figure 5D) and 6/15 mice were positive for intimal staining.

**Figure 2.** Accelerated lesion development is accompanied by more complex lesions in Atthsq1 congenic mice. Aortic root cross-sections from B6-Ldlr$^{-/-}$ (b/b) and congenic mice carrying one (b/m) or two (m*/m*) copies of the MOLF donor interval following 6-week WTD feeding. NC indicates necrotic core; arrow, fibrous cap; dotted arrow, intraplaque connective tissue.

**Figure 3.** Increased abundance of lesional versican in Atthsq1 congenics. A through D, Versican immunostaining (brown) in the aortic root of B6-Ldlr$^{-/-}$ (b/b) and congenic (m*/m*) mice fed WTD for 6 or 12 weeks. PI indicates atherosclerotic plaque; M, media. 400× magnification. E, Quantification by morphometric analysis. ANOVA, square root transformation (b/m groups not shown).
Versican has been indirectly implicated in the pathogenesis of vascular diseases. In atherosclerosis, versican is prominent in the ECM of early intimal thickenings as well as advanced lesions. It is also prominent in restenotic lesions after angioplasty. Versican binds LDL particles with high affinity, and it has been speculated that accumulation of versican in the vessel wall may promote both extracellular lipoprotein retention and intracellular uptake leading to foam cell formation. Versican also binds hyaluronan, forming expanded viscoelastic matrices required for SMC proliferation and migration in cell culture. Migration of SMCs from the media into the intima occurs early in atherogenesis and promotes lesion progression. Versican-hyaluronan structures also promote monocyte aggregation and adhesion via interaction with cell surface receptors including CD44, and L- and P-selectins. Thus, accumulation of versican may provide a local niche for rapid expansion of atherosclerotic plaques via multiple mechanisms.

Although prominent in human atherosclerotic lesions, versican does not appear to be a major matrix component in commonly-used mouse models of atherosclerosis. Kunjathoor and colleagues reported little to no accumulation of versican in early, intermediate, and advanced lesions of both B6-Ldlr−/− and B6-Apoε−/− mice. Our study is consistent with this earlier work as lesions derived from noncongenic B6-Ldlr−/− mice had little versican accumulation (Figure 3). Thus, the prominent accumulation of aortic versican observed in Athsq1 congenic strains was surprising and suggests a causative role determining disease susceptibility in this model. Versican accumulation was not seen in prelesional aortas of the congenic strain, indicating that there is a differential response to the atherogenic stimulus in congenics compared to controls. Our findings suggest that the MOLF-derived gene(s) underlying Athsq1 acts to posttranscriptionally regulate versican accumulation during lesion development.

The original linkage peak for Athsq1 (between D4Mit54 at 66 cM and D4Mit127 at 77 cM) was distal to the introgressed region carried by the m*/m* congenic mice. However, the m*/m* interval overlaps the 2-LOD-unit confidence interval for the QTL (57 to 97 cM) (Figure 1A). It is possible that the original QTL was detected because of atherogenic effects of more than one gene. However, the atherosclerotic phenotype

![Figure 4.](image)

**Figure 4.** Increased versican protein, not mRNA synthesis, in aortic homogenates from Athsq1 congenic mice. A, Immunoblot using an antibody for versican intact core protein. b/b indicates noncongenic; m*/m*, congenic. B, Quantification by densitometry. C, Relative abundance of versican mRNA in m*/m* aortas compared to b/b controls. 6-week WTD. t test, nontransformed data.

(1% to 5% of lesion area) compared to none of the mice receiving control BM. These results suggest that BM-derived cells play an important role in development of the lesion susceptibility phenotype, including versican accumulation in lesions.

**Discussion**

We have confirmed the atherosclerosis susceptibility QTL, Athsq1 on mouse chr 4, in congenic strains. Homozygous congenic mice have a dramatic phenotype with up to 4.5-fold greater lesion area and prominent accumulation of aortic versican compared to controls. We have shown through BM transplantation that the effect of Athsq1 is mediated, at least in part, by BM-derived cells. The association of versican accumulation with accelerated atherogenesis in this model provides in vivo evidence supporting a pathogenic role for versican.

![Figure 5.](image)

**Figure 5.** Increased lesion area and versican accumulation in B6-Ldlr−/− mice transplanted with Athsq1 congenic BM. A, Lesion areas after 11-week WTD feeding. Horizontal bars represent group means. B and C, Immuno-staining for versican (red). PI indicates plaque; M, media. 40× magnification. D, Quantification by morphometric analysis. t test, square root transformation.
of the full m/m congenics was similar to that of the shorter m*/m* congenics (Figure 1B), suggesting that the main gene(s) is located within the m*/m* interval.

The congenic interval is large (648 genes) and any discussion about possible candidate genes is highly speculative. However, it is interesting to note that comparative mapping of mouse and human chromosomes revealed that the m*/m* congenic interval contains the homologous region to the human CHD locus on 9p21 (Figure 6). The human locus has been mapped to an interval between Cdkn2a and Dmrt1a; mouse homologues of these genes reside on chr 4 at 88.9 Mb (Cdkn2a, as taken from NCBI m37 mouse assembly; April 2007) and 89.3 Mb (Dmrt1a). Further studies, including fine-mapping of the congenic interval, are required to identify the gene(s) underlying the Athsq1 locus. However, this study illustrates the potential usefulness of the mouse genetics approach when used in conjunction with detailed lesion analysis to define the underlying pathophysiology.

Acknowledgments

We thank Kadesha Collins-Fletcher for early technical assistance with histology.

Disclosures

None.

References


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Methods

Protein and mRNA Quantification of Versican. Proximal aortas were collected from 6-wk WTD-fed mice, cleared of adipose tissue and flash-frozen. For protein, tissues were incubated in extraction buffer (4M guanidine HCL, 100mM sodium sulfate, 100mM Tris base, 2.5mM Na$_2$EDTA, 0.5% TX-100, pH 7.0) overnight at 4 °C. Tissue extracts were dialyzed against urea buffer (8M urea, 2mM EDTA, 50mM Tris base, 0.5% TX-100, pH 7.5) and proteins isolated by DEAE Sephacel chromatography. (REF) Isolated proteoglycans were ethanol precipitated, chondroitin ABC lyase (REF) digested, and electrophoresed on 4-12% gradient SDS-Page gels with 3.5% stacking gel. Proteins were transferred to nitrocellulose and probed with mouse β-gag antibody. Proteins were then visualized using enhanced chemiluminescence kit (Applied Biosystems, Foster City, CA). Relative abundance of versican, identified as three distinct bands in the region above the 250 kDa marker (REFx3) was determined by quantitative image analysis using Kodak 1D Image Analysis software (Kodak, Rochester, NY). Total RNA was extracted with RNA-BEE (Tel-test, Inc., Friendswood, TX) using a 200 µl capacity dounce homogenizer. Reverse transcription was carried out using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using Taqman Gene Expression Assay Mm_00490179_ml for mouse versican (detects all splice variants). mRNA assays were performed in triplicate and normalized to endogenous eukaryotic 18s (Taqman Gene Expression Assay Hs_99999901_sl).
Table S1. Traditional risk factor assessment in *Athsq1* congenic and non-congenic mice.

Data are mean ± SD.

<table>
<thead>
<tr>
<th>Genotype at <em>Athsq1</em></th>
<th>Diet(^2)</th>
<th>N</th>
<th>TC(^3) (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>nHDL-C (mg/dl)</th>
<th>GLUC (mg/dl)</th>
<th>BWT (gm)</th>
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<td>12</td>
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<td>70±17</td>
<td>1251±249</td>
<td>141±29</td>
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\(^1\) m/m mice carry a MOLF-derived interval of chromosome 4 from *D4Mit185-D4Mit70* or *D4Mit185-D4Mit54*.

\(^2\) WTD, Western-type diet fed for 7 weeks.

\(^3\) TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; nHDL-C, non-HDL cholesterol (TC-HDL-C); GLUC, glucose; BWT, body weight.
Figure S1. Mean lesion areas in Athsq1 congenic and non-congenic strains fed 12-wk (A) or 6-wk (B) WTD and grouped by sex. Data are mean ± SD.

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**Females**

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<thead>
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<td>5</td>
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<tr>
<td>P</td>
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**Males**

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<th>6±10</th>
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<tr>
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<td>7</td>
<td>14</td>
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<tr>
<td>P</td>
<td>NS</td>
<td>P&lt;0.0005</td>
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Figure S2. Sex-specific quantification of versican in atherosclerotic lesions derived from \textit{Athsq1} congenic (m\textasciicircum{}*/m\textasciicircum{}*) and non-congenic (b/b) mice fed WTD for 6 wks. Data are mean ± SD.

**Males**

![Graph showing versican-positive medial area for males](image)

**Females**

![Graph showing versican-positive medial area for females](image)
Figure S3. Relative expression of CD44 (the cell surface receptor for hyaluronan) and hyaluronase-1 and –2 transcripts in aortic homogenates derived from *Athsg1* congenic (m*/m*) and non-congenic (b/b) mice fed WTD for 6 wks. Data are mean ± SD. N = 10 mice/group. Methods are as given for “mRNA quantification of versican” with the following Taqman probe sets: Hyal1 Mm00476206_m1, Hyal2 Mm01230689_g1, and CD44 Mm01277164_m1.
Figure S4. Relative expression of hyaluronan synthase-1, 2, and 3 transcripts in aortic homogenates derived from Athsqa1 congenic (m*/m*) and non-congenic (b/b) mice fed WTD for 6 wks. Data are mean ± SD. N = 10 mice/ group. Methods are as given for “mRNA quantification of versican” with the following Taqman probe sets: Has–1: Mm00468496_m1, Has–2: Mm00515089_m1, and Has–3: Mm00515092_m1.
Figure S5. Relative content of macrophages and vascular smooth muscle cells in atherosclerotic lesions derived from Athsq1 congenic (m*/m*) and non-congenic (b/b) mice fed WTD for 6 wks. Positive immunostaining for macrophages (Mac-3) and smooth muscle cells (α-actin) is brown. P, plaque; M, media. Original magnification is 200x.