Effects of DNA Damage in Smooth Muscle Cells in Atherosclerosis

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ABSTRACT

Rationale: DNA damage and the DNA damage response (DDR) have been identified in human atherosclerosis, including in vascular smooth muscle cells (VSMCs). However, although double strand breaks (DSBs) are hypothesized to promote plaque progression and instability, in part by promoting cell senescence, apoptosis and inflammation, the direct effects of DSBs in VSMCs seen in atherogenesis are unknown.

Objective: To determine the presence and effect of endogenous levels of DSBs in VSMCs on atherosclerosis.

Methods and Results: Human atherosclerotic plaque VSMCs showed increased expression of multiple DDR proteins in vitro and in vivo, particularly the MRN complex (MRE11, RAD50, NBS1) that senses DSB repair. Oxidative stress-induced DSBs were increased in plaque VSMCs, but DSB repair was maintained. To determine the effect of DSBs on atherosclerosis, we generated two novel transgenic mice lines expressing NBS1 or C-terminal deleted NBS1 only in VSMCs, and crossed them with ApoE−/− mice. SM22α-NBS1/ApoE−/− VSMCs showed enhanced DSB repair and decreased growth arrest and apoptosis, whereas SM22α-(ΔC)NBS1/ApoE−/− VSMCs showed reduced DSB repair and increased growth arrest and apoptosis. Accelerating or retarding DSB repair did not affect atherosclerosis extent or composition. However, VSMC DNA damage reduced relative fibrous cap areas, whereas accelerating DSB repair increased cap area and VSMC content.

Conclusions: Human atherosclerotic plaque VSMCs show increased DNA damage including DSBs and DDR activation. VSMC DNA damage has minimal effects on atherogenesis, but alters plaque phenotype inhibiting fibrous cap areas in advanced lesions. Inhibiting DNA damage in atherosclerosis may be a novel target to promote plaque stability.

Keywords: DNA damage, atherosclerosis, apoptosis, atherogenesis.

Nonstandard Abbreviations and Acronyms:
ATM ataxia telangiectasia mutated protein
ATR ATM-related kinase
DDR DNA damage response pathway
DSB double-stranded DNA break
MRN MRE11/RAD50/NBS-1 complex
SSB single-stranded DNA break
VSMCs Vascular smooth muscle cells
INTRODUCTION

Atherosclerosis is characterized by DNA damage. DNA damage is seen in early lesions, increases with disease severity, and is almost universal in advanced plaques. DNA damage includes both single- or double-stranded breaks (SSBs or DSBs), deleted sections of DNA, nucleotide modifications, and extrusions of nuclear DNA (micronuclei) (reviewed in 2). Vascular smooth muscle cells (VSMCs), endothelial cells, and macrophages all show both nuclear and mitochondrial DNA damage, as do circulating cells in patients with atherosclerosis. However, while DNA damage may affect function in all these cell types, VSMCs are long-lived cells in plaques and show persistent DNA damage, which may reflect both ongoing damage-inducing stimuli and/or defects in DNA repair.

DNA damage activates the DNA damage response (DDR) pathway, a hierarchical cascade of activated proteins that act as DDR sensors and effectors, to stall the cell cycle allowing DNA repair, to directly promote repair, or to induce cell senescence or apoptosis if damage is severe. DSBs are the most severe form of DNA damage and cause rapid recruitment of the MRE11/RAD50/NBS-1 (MRN) DNA sensor complex to break sites. MRN also promotes early DSB processing via DNA binding and nuclease activities, and recruits ataxia telangiectasia mutated protein (ATM) to DSBs, followed by ATM activation (reviewed in 9). Phosphorylated ATM and the histone H2AX accumulate at DSBs, flanking the site of DNA damage. γ-H2AX facilitates local assembly of checkpoint and DNA repair factors, and is a robust marker of DSBs. ATM and similar kinases including ATM-related kinase (ATR) orchestrate the DDR via activation of multiple downstream effectors including the cyclin-dependent kinase inhibitors p16 and p21, and the tumor suppressor gene p53. These effector proteins ultimately mediate growth arrest, cell senescence, or apoptosis.

The presence and extent of DNA damage and DDR markers in atherosclerotic plaques strongly support the view that DNA damage is detrimental in atherosclerosis. In particular, VSMCs in advanced plaques show low proliferation rates, increased senescence markers, and apoptosis. These features together with both DNA damage and DDR activation markers persist in cultured plaque VSMCs, suggesting that endogenous DNA damage may cause their growth arrest and apoptosis. However, studies on the direct effect of DNA damage on atherosclerosis are conflicting. For example, DNA damage induced by radiotherapy can either enhance or inhibit atherosclerosis and neointima formation in human or animal arteries (reviewed in 2). Some genetic diseases associated with DNA damage such as Hutchison Gilford Progeria (HGPS) and Werner syndromes show enhanced atherosclerosis, but many others do not (reviewed in 2). Similarly, genetic or pharmacological targeting of the DDR either inhibits or enhances atherosclerosis. Finally, whether these extensive experimental or natural germline changes recapitulate more modest DNA damage seen in human atherosclerosis is unclear.

The direct consequences of DNA damage in VSMCs in atherosclerosis are also unknown. Reduced VSMC proliferation and premature senescence might inhibit plaque growth in vivo, although secretion of multiple pro-inflammatory cytokines during senescence might promote atherosclerosis. VSMC apoptosis promotes atherogenesis, progression of established plaques and a vulnerable plaque phenotype, characterised by a thin fibrous cap and large necrotic core. However, VSMC apoptosis is induced by multiple pathways in atherosclerosis, and whether DNA damage promotes VSMC apoptosis in atherosclerosis is unknown.

We examined DNA damage/repair and DDR protein expression in human VSMCs derived from normal aorta and atherosclerotic plaques, and the effect of accelerating or retarding the DDR in VSMCs both in vitro and in novel transgenic mice in atherosclerosis. Human plaque VSMCs showed increased DSBs and constitutive DDR activation, particularly of pathways that sense and repair DSBs, but could repair DSBs efficiently. Accelerating or retarding the DDR in VSMCs changed advanced plaque phenotype, indicating that DNA damage may promote plaque instability.
METHODS

**Human atherosclerotic plaque and normal vessels.**
Human tissue was obtained under informed consent using protocols approved by the Cambridge or Huntingdon Research Ethical Committee. Atherosclerotic plaques and normal aorta were obtained from separate patients undergoing carotid endarterectomy or coronary artery bypass/valve replacement respectively. However, there was no difference in mean age or gender of donors of aorta vs. carotid samples. VSMCs were cultured from explants as described before and Online, studied at passages 2–5; VSMC cultures from individual patients were not pooled.

**PCR Arrays.**
PCR Arrays specific for 84 human DNA damage signaling pathway genes (PAHS-029) were performed according to the manufacturer’s instructions (and Online). NBS1, MRE11, and RAD50 expression in additional plaque (n=3) or normal (n=3) VSMC cultures was confirmed using gene-specific Taqman primer probes (Applied Biosystems/Life Technologies).

**Human immunohistochemistry.**
Immunohistochemistry on human vessels was as described before. Primary antibodies were against mouse SMA (DAKO 0.014mg/L/1:50), NBS1 (Abcam 47386 2 mg/ml), H2AX (NEB 2577 1:1000), MRE11 (NEB 4895 1:1000), and RAD50 (Sigma R1653 1mg/ml), or isotype-matched controls.

**DNA damage mediator analysis.**
DNA damage mediators were examined by Western blot as described previously and Online.

**Immunocytochemistry.**
Nuclear foci of γ-H2AX and P-ATM were examined as previously described and Online.

**Comet assay.**
Comet assay was performed using 3x10^4 human VSMCs/well or 5x10^4 mouse VSMCs/well as described previously and Online.

**Generation of transgenic mice.**
All animal experiments were approved under UK Home Office licensing. Transgenic mice were generated by pro-nuclear injection into FVB/B16 hybrids, and positive progeny crossed with littermates to generate individual lines. Genotyping for NBS1, NBS1(ΔC) homo- and heterozygosity was performed as described Online.

**Mouse immunohistochemistry.**
Immunohistochemistry of mouse tissue was performed as described previously, and online.

**Mouse VSMC culture.**
Mouse VSMCs were isolated and cultured as previously described.

**Time-lapse videomicroscopy.**
Mouse VSMCs were analysed for proliferation and apoptosis by time-lapse videomicroscopy as described before.

**Atherosclerosis protocols.**
ApoE^−/−, SM22α−NBS1/ApoE^−/−, and SM22α−(ΔC)NBS1/ApoE^−/− male and female littermate mice were fed High Fat Diet (HFD-21% total fat, 0.2% cholesterol, 0% sodium cholate) from 6-20w. Blood was taken every 4w, and serum lipids analysed for total cholesterol, triglycerides, LDL and HDL using a Dade-

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Behring Dimension autoanalyzer and LDL calculated using the Friedwald formula. Blood pressure was analysed by tail cuff.

**Atherosclerosis analysis.**
Atherosclerosis extent and composition was analyzed as described previously\(^{22}\) and in the online data supplement.

**Statistical analysis.**
Comparison between three groups of mice was conducted using a one-way ANOVA. Statistical analysis was performed using Student’s \(t\)-test for data following a normal distribution. Data presented are means\(\pm\)SEM with significance \(p<0.05\).

**RESULTS**

*Human atherosclerotic plaque VSMCs show enhanced DNA damage and increased expression of MRN components.*

To examine baseline DDR signaling in VSMCs, we cultured normal human aortic (\(n=6\)) or plaque (\(n=4\)) VSMCs from individual patients. DDR activation was examined using duplicate PCR arrays specific for 84 human DNA damage signaling pathway genes. To examine heterogeneity between cultures from the same source (aortic or plaque) we first compared gene expression relative to 5 housekeeping genes (Online Figure I). Heatmaps demonstrated surprisingly limited heterogeneity within aortic or plaque VSMCs, with <2-fold variation in specific gene expression between individual patient cultures (Online Figure I). However, compared to aortic VSMCs, 19/84 genes were upregulated >1.5-fold in plaque VSMCs, and 25/84 downregulated >1.5-fold (Online Table I). Upregulated genes included those involved in DSB repair (MRE11, RAD50), base excision repair (OGG1, MUTYH, NTHL1), DNA excision repair (XPA), mismatch repair (MLH3, MUTYH, TP73, TREX1), and chromatin remodeling (ATRX), with some known p53 target genes (PCBP4, GADD45A, GML) or homologs (TP73) and antiproliferative genes (BTG2, GADD45A). This suggests that plaque VSMCs exhibit multiple forms of DNA damage, with consequent upregulation of DDR proteins associated with multiple repair pathways.

We used MetaCore network analysis software to determine common upstream pathways that were activated in plaque VSMCs. Although individual components of multiple pathways were upregulated, DSB sensing was the most consistent pathway activated in plaque VSMCs, including MRN complex proteins and ATM (Figure 1A). Basal levels of MRE11, RAD50, and ATM were upregulated 2.2, 2.4, and 2.6-fold respectively in cultured plaque VSMCs on the array (Online Table I), although relative NBS1 expression was 1.3-fold, which did not reach significance. qPCR was used to verify increased expression of MRE11 (3.1-fold), RAD50 (2.2-fold) and NBS1 (1.7-fold) in separate cultures of human plaque (\(n=3\)) and normal aortic (\(n=3\)) VSMCs. To determine whether these mRNA differences in culture reflect protein differences in vivo, we examined their expression and the DSB marker \(\gamma\)-H2AX in human plaques (\(n=5\)) and normal aorta (\(n=3\)) by immunohistochemistry, co-localised with \(\alpha\)-SMA to identify VSMCs. Plaques showed significantly increased % of VSMCs expressing MRE11, RAD50, NBS1, and \(\gamma\)-H2AX (Figure 1B-C).

*Plaque VSMCs show increased formation of DDR protein foci and DNA damage.*

Sensor proteins are recruited to DNA damage sites and orchestrate formation of multi-protein complexes for DDR activation. NBS1 is an initial DSB sensor, forming a trimeric complex with MRE11 and RAD50 (reviewed in\(^{24}\)) that tethers the MRN complex to DNA damage sites. MRN translocates to the nucleus and binds at DSBs to form distinct foci with H2AX that are phosphorylated at Ser\(^{139}\) following...
DNA damage\textsuperscript{25}. We therefore examined nuclear foci of $\gamma$-H2AX and P-ATM as markers of a DSB-induced DDR in normal aortic and plaque VSMCs after treatment with the oxidant t-BHP. Although multiple agents generate DNA damage within atherosclerotic plaques, including oxidative stress, modified lipids and cytokines, t-BHP is a highly reproducible agent to induce DSBs in VSMCs\textsuperscript{1,22}. $\gamma$-H2AX and P-ATM increased and then declined after t-BHP treatment in normal VSMCs; however, plaque VSMCs showed increased $\gamma$-H2AX and P-ATM foci at baseline and after t-BHP, suggesting both increased baseline DDR activation and a robust DDR induction through ATM (Online Figure II).

We next utilised single cell alkaline electrophoresis (comet assay) to determine both baseline DSBs and kinetics of DNA damage and repair in human VSMCs. The initial increase in comet tail length/moment measures sensitivity to DNA damage and efficiency of rapid repair, while time to recovery of baseline comet tail length/moment after a single damage stimulus indicates late repair. Human aortic VSMCs showed rapid induction of DSBs after treatment with t-BHP; repair was also rapid (>50\% repair by 1h) and complete by 4h (Figure 1D, Online Figure III). Plaque VSMCs showed a similar rapid induction and repair of DSBs (>50\% repair by 1h); initial DNA damage was increased in plaque compared with aortic VSMCs, but returned to similar levels by 2h (Figure 1D). Thus, human plaque VSMCs show persistent DNA damage including DSBs and a DDR and increased sensitivity to DSB-inducing agents, but retain rapid initial DSB repair.

\textit{NBS1 regulates DNA damage and repair in VSMCs.}

NBS1 contains a fork-head associated (FHA) and two BRCA1 C-terminal (BRCT) domains required for protein–protein interactions (including binding to mediator of the DNA damage checkpoint 1 (MDC1))\textsuperscript{26}. The C-terminus of NBS1 also contains a MRE11 binding domain and a conserved sequence motif that binds and recruits ATM to damaged DNA, where it is subsequently autophosphorylated\textsuperscript{27}; this ATM/NBS1 interaction is essential for ATM recruitment to DNA and activation\textsuperscript{28}. The C-terminus of NBS1 is not required for ATM phosphorylation \textit{per se}, but is important for phosphorylation of ATM targets, as NBS1(ΔC) is unable to support ATM-mediated signaling events that trigger cell cycle checkpoints and DNA repair\textsuperscript{27}.

The increased expression of MRN components in plaque VSMCs (Figure 1A-C) together with increased DSB induction but rapid initial DSB repair (Figure 1D) suggest the different DNA damage and repair kinetics in plaque VSMCs may be regulated through MRN complex binding to DSBs. Indeed, we found previously that NBS1 regulates DSB repair kinetics in human VSMCs pre-treated with atorvastatin, whereas MRE11 or RAD50 expression was unaffected\textsuperscript{1}. To examine the effects of accelerating or inhibiting DSB repair in VSMCs, we therefore generated two novel transgenic mouse lines that constitutively express human NBS1 or NBS1 lacking the C-terminus (ΔC) from the minimal SM22α promoter (Figure 2A), with the expectation that NBS1 overexpression would accelerate DSB repair and NBS1(ΔC) delay repair. The minimal SM22α promoter is expressed only in large artery SMCs in adult mice\textsuperscript{29}, and is thus highly specific for VSMCs. We also deleted its specific G/C repressor region to avoid losing transgene expression when VSMCs undergo phenotypic modulation in culture or in atherosclerosis\textsuperscript{30}.

SM22α–NBS1 or SM22α(ΔC)NBS1 mice were born with expected frequencies, transmitted the transgene normally and had no gross phenotype. 2 founders from each line were studied which showed identical expression and transmission. Mice were backcrossed 5 times to ApoE\textsuperscript{−/−} mice, which did not affect transgene expression or transmission. RTPCR confirmed expression of human NBS1 or NBS1(ΔC) in aortas of transgenic but not control ApoE\textsuperscript{−/−} littermates, which was absent in small intestine or liver of transgenic mice (Figure 2B-D). Transgene expression was maintained in VSMCs cultured from SM22α-NBS1/ApoE\textsuperscript{−/−} or SM22α(ΔC)NBS1/ApoE\textsuperscript{−/−} mice by both RTPCR (Figure 2E) and Western blot (Figure 2F), demonstrating promoter activity even after phenotypic modulation.
We next examined the effect of NBS1 and NBS1(ΔC) expression on arterial structure and DNA damage. The structure and medial cellularity of aortas in SM22α-NBS1/ApoE−/− and SM22α-(ΔC)NBS1/ApoE−/− mice was similar to Control ApoE−/− mice (Figure 3A). However, γ-H2AX was expressed in <1% of VSMCs in Control ApoE−/− or SM22α-NBS1/ApoE−/− aortas, but was seen in 14.3±0.7% of VSMCs in SM22α-(ΔC)NBS1/ApoE−/− mice (Mean±SEM, n=5). P-ATM and p21 were both expressed in some VSMCs in Control mice; however, their expression was reduced in SM22α-NBS1/ApoE−/−, and increased in SM22α-(ΔC)NBS1/ApoE−/− mice compared to Controls (Figure 3A-B). No apoptotic VSMCs were detected by TUNEL in any mouse groups. Thus, NBS1(ΔC) expression in vivo increases DNA damage and DDR activation, but not sufficient to induce apoptosis; in contrast, NBS1 expression reduces basal DDR activation in vivo.

NBS1 and (ΔC)NBS1 regulate DDR signaling in VSMCs.

To determine the consequences of NBS1 or NBS1(ΔC) expression on VSMCs, we examined DDR activation after t-BHP treatment through formation of nuclear foci on DSBs. γ-H2AX foci intensity increased after 30 min of t-BHP treatment (Time 0), but was similar in all cell types (Figure 4A), indicating that H2AX recruitment and phosphorylation on DSBs is unaffected by differences in NBS1 expression. Nuclear P-ATM expression increased after t-BHP treatment, but again was similar in all cell types (Figure 4B). In contrast, consistent with the known role of NBS1 and its C-terminus 27, colocalization of P-ATM with γ-H2AX was reduced in SM22α-(ΔC)NBS1/ApoE−/− VSMCs (Figure 4C), indicative of reduced P-ATM recruitment to sites of DNA damage. Immunoprecipitation studies confirmed impaired ATM binding to NBS1 in VSMCs expressing NBS1(C) compared with VSMCs expressing NBS1 (Online Figure IV).

The downstream consequences of DDR activation are induced by signals from sensors, particularly γ-H2AX and the ATM/ATR apical kinases that are phosphorylated in response to DNA damage detected by MRN, or through autophosphorylation. ATM and ATR have multiple targets that signal growth arrest, cell senescence, and apoptosis, but the C-terminus of NBS1 is required for ATM target phosphorylation and effective DDR transmission 27. For example, NBS1 phosphorylation is associated with increased MRN activity as MRE11 binding to the NBS1 C-terminal is required for MRN nuclear localization 27. In contrast, MRE11 phosphorylation is dependent upon NBS1 31, and is associated with reduced MRN activity through reduced interaction between the MRN complex and chromatin 32.

We therefore examined expression and phosphorylation of H2AX, both specific ATM substrates (NBS1 and MRE11) and generic ATM/R substrates, and downstream effector proteins including p53 and p21 after t-BHP treatment (Figure 4D). γ-H2AX, the initial DSB sensor, was induced similarly in control and SM22α-NBS1(ΔC) VSMCs, but SM22α-NBS1/ApoE−/− VSMCs showed increased expression after t-BHP (Figure 4D). P-NBS1 was seen basally in control VSMCs, increased early (within 30 min of t-BHP treatment), and returned to normal by 120 min. P-NBS1 was increased in SM22α-NBS1/ApoE−/− compared with control ApoE−/− VSMCs, and peaked at 30 min of recovery; in contrast, P-NBS1 was not detectable at any point in SM22α-(ΔC)NBS1/ApoE−/− VSMCs. MRE11 phosphorylation was increased at 30 and 120 min after t-BHP in control and SM22α-NBS1(ΔC) VSMCs, but suppressed in SM22α-NBS1/ApoE−/− VSMCs. Phosphorylation at the ATM/ATR substrate motif was induced in control ApoE−/− and SM22α-NBS1(ΔC) VSMCs at 30 min and 120 min of recovery, whereas ATM/ATR activity was increased in SM22α-NBS1 VSMCs at all time points (Figure 4D). In control VSMCs both p53 phosphorylation and p21 induction were relatively late events (120 and 30 min into recovery respectively), suggesting that they reflect continued DNA damage. Both P-p53 and p21 were suppressed in SM22α-NBS1/ApoE−/− VSMCs, suggesting more rapid repair. These changes in DDR protein expression suggest that ectopic NBS1 increases MRN complex activity and H2AX phosphorylation with subsequent increased ATM/R activity.
and more rapid repair, whereas NBS1ΔC reduces P-ATM localisation to DNA damage sites, and decreases both MRN and ATM/R activities slowing repair.

**NBS1 and NBS1(ΔC) regulate DNA damage and apoptosis in VSMCs.**

To determine the downstream consequences of these different DDR signals, we examined the effects of NBS1 or NBS1(ΔC) on DSB induction and repair in VSMCs. Similar to normal human VSMCs, control ApoE−/− VSMCs showed increased comet tail moments after t-BHP treatment, with rapid repair and return to baseline by 3h recovery (Online Figure V, Figure 5A). DSBs were reduced in SM22α-NBS1/ApoE−/− compared with control VSMCs; in contrast, SM22α-(ΔC)NBS1/ApoE−/− VSMCs showed increased DSBs on induction but still with rapid initial repair, a pattern similar to human plaque VSMCs (Figure 1D).

These different kinetics of DNA damage and repair would be expected to have different effects on DNA damage-induced growth arrest and apoptosis. Indeed, t-BHP caused rapid inhibition of cell proliferation in control ApoE−/− VSMCs; this was markedly reduced in SM22α-NBS1/ApoE−/−, but not in SM22α-(ΔC)NBS1/ApoE−/− VSMCs (Figure 5B). t-BHP significantly increased apoptosis in ApoE−/− VSMCs; t-BHP-induced apoptosis was increased further in SM22α-(ΔC)NBS1/ApoE−/− VSMCs, but reduced in SM22α-NBS1/ApoE−/− relative to control VSMCs (Figure 5C).

**Expression of NBS1 and NBS1(ΔC) in VSMCs does not affect plaque size.**

These studies indicate that NBS1 expression in VSMCs increases DDR signaling from ATM/R after DNA damage, reduces DSBs, and protects against DNA-damage-induced growth arrest and apoptosis, whereas NBS1(ΔC) inhibits P-ATM recruitment to DSBs, inhibits MRN and ATM/R activity, increases DSBs after t-BHP, and cannot protect against DNA damage-induced arrest and apoptosis. Thus, SM22α-NBS1/ApoE−/− and SM22α-(ΔC)NBS1/ApoE−/− mice represent an experimental system to determine how increasing or decreasing endogenous VSMC DNA damage affects atherosclerosis.

Littermate male and female Control ApoE−/−, SM22α-NBS1+/+/ApoE−/− and SM22α-NBS1(ΔC)+/+/ApoE−/− mice were fed a high-fat diet from 6-20w. Serum LDL, HDL, triglycerides and total cholesterol, body weights and blood pressure were similar between mouse groups (Online Figure VI). Aortic root and brachiocephalic plaques were examined for atherosclerosis extent and composition. Surprisingly, despite marked effects on VSMC DNA damage, DSB extent, DDR signaling, growth arrest and apoptosis in vitro, there was no significant difference in aortic root plaque area between mouse groups either in absolute terms or relative to aortic root area (Figures 6,7, Table 1). Brachiocephalic % plaque areas were also similar between mouse groups (Online Figure VII, Online Table II). % VSMCs and macrophage areas were similar in both aortic root and brachiocephalic plaques, although necrotic core area was slightly reduced in SM22α-NBS1(ΔC)+/+/ApoE−/− mice aortic plaques (Figures 6,7, Online Figure VIII, Table 1, Online Table II).

Although DNA damage is seen predominantly in advanced plaques, it is possible that protective effects of NBS1 overexpression on endogenous DNA damage in atherogenesis might be seen at earlier time points. We therefore examined plaques at 14w (8w fat feeding). Similar to 20w plaques, there was no difference in plaque area in SM22α-NBS1+/+/ApoE−/− vs. control mice either in absolute terms or relative to vessel area at 14w, and no difference in VSMC or macrophage contents (Online Table III).
VSMC DNA damage reduces fibrous cap area.

Increased DNA damage causing reduced VSMC proliferation and increased apoptosis would be predicted to induce features of vulnerable plaques, including smaller fibrous caps with reduced VSMC content, and inflammation. We therefore undertook a detailed analysis of aortic root and brachiocephalic plaques at 20w. SM22α-NBS1/ApoE−/− mice aortic plaques had increased absolute fibrous cap areas, and both aortic and brachiocephalic plaques had increased cap VSMC content; in contrast, relative cap area (fibrous cap:plaque ratio) was reduced in aortic plaques in SM22α-(ΔC)NBS1/ApoE−/− mice, and increased in SM22α-NBS1/ApoE−/− mice brachiocephalic plaques (Figures 6, 7, Table 1, Online Table II). Aortic plaque γ-H2AX expression in VSMCs was increased in SM22α-NBS1(ΔC)/ApoE−/− mice, but decreased in SM22-NBS1+/−/ApoE−/− mice compared with control ApoE−/− mice. Although in SM22−/−NBS1/ApoE−/− mice there was a trend for increased proliferation (aortic plaques) and reduced apoptosis (aortic and brachiocephalic plaques), overall VSMC proliferation and apoptosis were similar between groups (Online Figure VIII, Table 1, Online Table II). Although NBS1 is also important for survival after oxidative stress, ROS levels were similar in cultured VSMCs (Online Figure IX) and 8-oxo-G-positive VSMCs were similar in aortic plaques in vivo in all 3 groups (Table 1).

DISCUSSION

Fat feeding of rabbits or ApoE−/− mice results in DNA damage and DDR activation, including appearance of oxidative lesions, DNA strand breaks, up-regulation of DNA repair enzymes, and DDR markers. Similarly human plaques show widespread oxidative DNA damage and strand breaks, with activation of multiple DNA repair pathways, including base excision and non-specific repair. While many cell types show DNA damage in plaques, a number of factors indicate that DNA damage-induced dysfunction is particularly important in VSMCs, and may directly affect both atherogenesis and plaque stability. For example, VSMCs in human plaques show DNA damage and DDR markers from AHA Stage II onwards, including phosphorylation of H2AX and ATM/R substrates; damage markers increase with disease severity and become nearly universal in VSMCs in advanced plaques. VSMCs cultured from human plaques retain DNA damage and DDR activation (and shown here) including DSBs, the most severe form of DNA damage. Human plaque VSMCs show characteristic features in culture consistent with DNA damage, including poor proliferation, early senescence and increased apoptosis, and VSMC senescence and increased apoptosis are also evident in vivo. However, although these findings implicate DNA damage as inducing VSMC dysfunction in advanced atherosclerosis, to date we have no direct evidence that levels of DNA damage found in VSMCs during atherosclerosis (as opposed to those occurring in DDR disorders or by external sources) have any functional effect. We also do not know whether enhancing or reducing endogenous DNA damage and the DDR seen in atherosclerosis affects plaque development or composition.

Using PCR arrays we find that human plaque VSMCs show differential expression of genes from multiple DNA damage and repair pathways, most likely reflecting stimuli such as free radicals and oxidised lipids that induce different forms of DNA damage. However, pathway analysis demonstrates that the most consistent changes in plaque VSMCs are up-regulation of DSB sensing proteins including the MRN complex, and increased expression of MRN components in plaque VSMCs was confirmed both in vitro and in vivo. Plaque VSMCs showed increased DDR activation at baseline, and both increased DDR activation and DSBs after oxidative stress in culture. Increased DSBs and MRN expression might be due to increased exposure to damage-inducing stimuli, and/or a defect in DSB repair. However, initial DSB repair was both rapid and as efficient in plaque as in normal VSMCs, suggesting that differences in MRN expression and DDR activation in atherosclerosis are likely to be compensatory rather than detrimental.
To determine the effects of accelerating or inhibiting DSB repair in VSMCs in vitro and in vivo, we generated two novel transgenic mouse lines that expressed NBS1 or an ATM activation-defective form of NBS1 (NBS1(ΔC)) from the VSMC-specific minimal SM22α promoter. In vitro, NBS1 expression increased phosphorylation of both H2AX and ATM/R substrates, including the ATM target NBS1 itself, and reduced DSBs, growth arrest and apoptosis after oxidative stress. This further suggests that increased MRN component expression seen in human plaque VSMCs, and increased ATM phosphorylation seen in earlier studies, is compensatory and beneficial. In contrast, NBS1(ΔC) expression impaired P-ATM co-localization to DSBs, reduced ATM/R activity, and reduced NBS1 phosphorylation compared with NBS1 VSMCs. NBS1(ΔC) expression increased DSBs in VSMCs, and did not protect against oxidative stress-induced growth arrest or apoptosis. NBS1(ΔC) expression also increased DNA damage and DDR markers in mouse aortas at baseline. Therefore, these mice represent mechanisms of accelerating and inhibiting the DDR after DSBs in VSMCs, and manipulating endogenous levels of DNA damage in vivo.

We analyzed the effects of VSMC-specific NBS1 or NBS1(ΔC) expression on atherogenesis and morphology of advanced plaques in ApoE−/− mice. Despite marked effects of NBS1 and NBS1(ΔC) on VSMCs in vitro, and increased or decreased DNA damage in plaques in mice expressing NBS1(ΔC) or NBS1 respectively, neither NBS1 nor NBS1(ΔC) affected plaque extent in two separate vascular beds, and NBS1 expression had no effect on plaque size, or overall VSMC or macrophage content at two different time points.

We do not think the lack of effect on plaque area in the transgenic mice is due to inadequate expression of MRN proteins or inadequate augmentation/suppression of pathways involved in DSB repair. VSMCs from these mice maintained transgene expression in vitro and in vivo. In vitro, NBS1 or NBS1(ΔC) VSMCs had marked differences in DDR signaling, a robust decrease or increase in DSB formation respectively, and either inhibition (NBS1) or augmentation NBS1(ΔC) of DNA damage-induced growth arrest and apoptosis. In vivo in normal aortas, NBS1 decreased P-ATM and p21 expression in VSMCs, while NBS1(ΔC) increased their expression and γ-H2AX. In vivo in atherosclerosis, NBS1(ΔC) and NBS1 increased or decreased γ-H2AX expression in plaques respectively. Rather, we suspect that VSMC apoptosis is a more critical determinant of atherosclerotic plaque size than DNA damage. VSMC apoptosis accelerates atherogenesis and progression of established plaques markedly. In contrast, the increased DNA damage in SM22α-NBS1(ΔC)/ApoE−/− mice was insufficient to induce VSMC apoptosis in vivo, and apoptosis was not suppressed in SM22α-NBS/ApoE−/− mice. Thus, we suspect that VSMC DNA damage only accelerates atherogenesis if sufficient to induce apoptosis, and that plaque VSMCs repair sublethal DSBs sufficiently well not to compromise function. This hypothesis is consistent with our finding of rapid and efficient DSB repair in human plaque VSMCs, and a previous study showing that markers of DNA damage and repair did not correlate with apoptosis.

Our study also does not exclude the possibility that DNA damage in VSMCs above the levels normally seen in atherosclerosis can affect atherogenesis, or that DSBs can affect plaque progression rather than initiation. For example, local breast or thyroid irradiation is associated with late development of coronary or carotid atherosclerosis respectively. However, the extent of DNA damage is likely to determine the subsequent consequences. Increasing doses of radiation promote atherosclerosis in ApoE−/− mice, particularly an inflammatory and thrombotic phenotype, whilst intracoronary brachytherapy was used to prevent or treat in-stent stenosis. Furthermore, irradiation also affects multiple cell types and studies from brachytherapy and radioactive stents show that endothelial cells are very sensitive to DNA damage and apoptosis.

In contrast, DNA damage is most prominent in advanced plaques in humans, and we find that the major effects of DSBs in VSMCs appear in advanced plaques. NBS1(ΔC) expression increased DNA damage and reduced relative fibrous cap area, whereas NBS1 expression reduced DNA damage and
increased both absolute cap area and VSMC content. DNA damage and increased DDR signaling seen in plaque VSMCs in vitro and in vivo has provided a highly plausible explanation for multiple features of advanced plaque VSMCs, namely increased apoptosis, reduced cell proliferation and early senescence. Here we find that DSB repair determines sensitivity to DNA-damage induced growth arrest and apoptosis in vitro, but the major effects in vivo are on fibrous cap VSMCs where DNA damage can affect cap structure without demonstrable changes in apoptosis frequencies. Together with studies showing that senescence markers are most evident in cap VSMCs, this suggests that DNA damage-induced VSMC senescence may be an important pathological process in determining cap integrity. In contrast, VSMC apoptosis is triggered also by both death receptors and defective cell survival signaling, which may be more potent than DNA damage in atherosclerosis.

In conclusion, we find that human plaque VSMCs show increased basal DNA damage and enhanced induction of DSBs after oxidative stress, associated with increased expression of multiple DDR proteins. Inhibition of NBS1 signaling in VSMCs results in DSB repair profiles that resemble those seen in human plaque VSMCs, and marked effects in vitro on DDR signaling, cell proliferation and apoptosis. Neither accelerating nor retarding DSB repair in VSMCs affects plaque burden, but have marked effects on fibrous cap structure. Thus, inhibiting VSMC DNA damage in atherosclerosis may be a novel target to promote plaque stability.

SOURCES OF FUNDING
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DISCLOSURES
None.

REFERENCES


FIGURE LEGENDS

Figure 1. Human plaque VSMCs show enhanced DNA damage and expression of MRN components. (A) Pathway analysis of DDR pathways upregulated in plaque (n=4) vs. normal aortic (n=6) human VSMCs. (B) Immunohistochemistry for MRE11, RAD50, NBS1 or γ-H2AX (brown) co-localized with α-SMA (blue) in aorta or plaque. Scale bar: low power, 500μm (plaque) or 250μm (aorta); high power 50μm. (C) Quantification of MRE11, RAD50, NBS1, or γ-H2AX-positive VSMCs from aorta (n=3) or plaque tissue (n=5). (D) Comet assay at baseline (U), after t-BHP treatment for 30 minutes (t=0), or after t-BHP removal (1-6h recovery) in aortic or plaque VSMCs. Treatments were performed in triplicate and data are means pooled from three separate experiments; error bars represent SEMs.

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Figure 5. Expression of NBS1 or NBS1(ΔC) regulates DNA damage, growth arrest and apoptosis. (A) Mean comet tail moment following t-BHP treatment at baseline (untreated U) or after t-BHP treatment (40μM, 30 min of t-BHP), or after 0.5, 3, 5, 12 or 8 h of recovery from cells treated in triplicate. Data was pooled from three separate experimental analyses. (B) % VSMCs undergoing proliferation 10 h after t-BHP treatment (40μM, 30 min) relative to untreated cells. (C) % VSMCs undergoing apoptosis after t-BHP treatment. Data are means±SEM, n=3).

Figure 6. Expression of NBS1 or NBS1(ΔC) does not alter extent of atherosclerosis. Histochemistry and immunohistochemistry of aortic root plaques of Control ApoE−/−, SM22α-NBS1/ApoE−/− and SM22α-NBS1(ΔC)/ApoE−/− mice after 14 w of fat feeding. Sections were stained with Masson’s Trichrome or antibodies to α-SMA. Scale bar: low power, 500μm; high power,100μm.
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<table>
<thead>
<tr>
<th></th>
<th>Control ApoE&lt;sup&gt;−/−&lt;/sup&gt; n=15</th>
<th>SM22α-NBS1/ApoE&lt;sup&gt;−/−&lt;/sup&gt; n=11</th>
<th>SM22α-NBS1(ΔC)/ApoE&lt;sup&gt;−/−&lt;/sup&gt; n=12</th>
<th>1-way ANOVA</th>
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</thead>
<tbody>
<tr>
<td>Aortic Root Plaque Area (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>0.39±0.04</td>
<td>0.52±0.058</td>
<td>0.45±0.073</td>
<td>p=0.32</td>
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<tr>
<td>Aortic Root Plaque Area (%)</td>
<td>35.1±2.57</td>
<td>40.6±2.91</td>
<td>35.0±4.08</td>
<td>p=0.39</td>
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<td>SMA-positive Cells (%)</td>
<td>28.2±2.34</td>
<td>25.9±3.46</td>
<td>35.8±2.84</td>
<td>p=0.056</td>
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<td>MAC3-positive area (% Plaque)</td>
<td>20.3±2.46</td>
<td>19.9±2.12</td>
<td>25.1±4.32</td>
<td>p=0.45</td>
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<td>Necrotic Core area (% plaque)</td>
<td>32.3±2.58</td>
<td>38.2±3.33</td>
<td>24.3±3.60*</td>
<td>p=0.019</td>
</tr>
<tr>
<td>Fibrous cap area (μm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>66376±9496</td>
<td>92674±17338*</td>
<td>46299±6885</td>
<td>p=0.018</td>
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<tr>
<td>Cap/plaque area ratio</td>
<td>0.169±0.02</td>
<td>0.171±0.02</td>
<td>0.105±0.007*</td>
<td>p=0.005</td>
</tr>
<tr>
<td>SMA-positive cells in Fibrous cap (%)</td>
<td>9.8±0.89</td>
<td>14.79±1.1*</td>
<td>13.1±1.5</td>
<td>p=0.02</td>
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<tr>
<td>γ-H2AX-positive VSMCs (%)</td>
<td>4.8±0.80</td>
<td>2.7±0.36*</td>
<td>7.7±0.90*</td>
<td>p=0.003</td>
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<td>PCNA-positive VSMCs (%)</td>
<td>7.4±0.58</td>
<td>8.5±0.99</td>
<td>7.0±0.76</td>
<td>p=0.42</td>
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<td>TUNEL-positive VSMCs (%)</td>
<td>0.046±0.01</td>
<td>0.039±0.005</td>
<td>0.056±0.02</td>
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<tr>
<td>8-oxo-G positive VSMCs (%)</td>
<td>25.3±3.1</td>
<td>30.4±3.25</td>
<td>25.8±2.6</td>
<td>p=0.57</td>
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</tbody>
</table>

Table 1. Histological analysis of aortic root plaques in Control ApoE<sup>−/−</sup>, SM22α-NBS1/ApoE<sup>−/−</sup> and SM22α-NBS1(ΔC)/ApoE<sup>−/−</sup> mice. Data are means±SEM. *indicates statistical significance vs. control mice.
Novelty and Significance

What Is Known?

- Atherosclerosis is associated with DNA damage, which affects both resident cells in the vessel wall and circulating cells that migrate into plaque.
- However, the effects of DNA damage on plaque instability and growth are not well known.

What New Information Does This Article Contribute?

- Vascular smooth muscle cells (VSMCs) from human atherosclerotic plaques show activation of multiple DNA repair pathways.
- DNA damage in VSMCs promotes plaque instability, but has little effect on plaque growth.

Markers of DNA damage increase with advancing atherosclerosis, with extensive damage in clinically important plaques; however, whether DNA damage is cause or consequence of atherosclerosis, and what its functional effects might be are unclear. In particular, DNA damage can promote growth arrest, cell senescence and cell death. VSMCs are long-lived cells in the vessel wall, so that effects of DNA damage may be particularly important in VSMCs. We examined DNA damage and repair signaling pathways in VSMCs from human plaques and normal aorta. We found that multiple DNA repair pathways are activated in human plaque VSMCs, and in particular, that double-strand DNA breaks (DSBs) and their downstream signaling pathways (the most extensive form of damage), are increased in plaque VSMCs. To examine the effects of DNA damage, we generated mice that have accelerated or defective DSB repair, and studied them in a mouse model of atherosclerosis. We found that accelerating DSB repair improved markers of atherosclerotic plaque stability, whereas inhibiting DNA repair promoted instability, with marked effects on the fibrous cap. We conclude that inhibiting or preventing DNA damage in atherosclerosis may be a novel target to promote plaque stability.
Figure 1. Human plaque VSMCs show enhanced DNA damage and expression of MRN components

(A) Pathway analysis of DDR pathways upregulated in plaque (n=4) vs. normal aortic (n=6) human VSMCs

(B) Immunohistochemistry for MRE11, RAD50, NBS1 or γ-H2AX (brown) co-localized with α-SMA (blue) in aorta or plaque. Scale bar: low power, 500μm (plaque) or 250μm (aorta); high power 50μm

(C) Quantification of MRE11, RAD50, NBS1, or γ-H2AX-positive VSMCs from aorta (n=3) or plaque tissue (n=5).

(D) Comet assay at baseline (U), after t-BHP treatment for 30 minutes (t=0), or after t-BHP removal (1-6h recovery) in aortic or plaque VSMCs. Treatments were performed in triplicate and data are means pooled from three separate experiments; error bars represent SEMs.
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Effects of DNA Damage in Smooth Muscle Cells in Atherosclerosis
Kelly L Gray, Sheetal V Kumar, Nichola Figg, James Harrison, Lauren Baker, John R. Mercer, Trevor D Littlewood and Martin R Bennett

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Online methods

Culture of human VSMCs
Human VSMCs were cultured from explants. The endothelial layer was removed using a scalpel and tissue cut into small pieces (approximately 2-3 mm²), placed into 6-well plates containing 1 ml media (DMEM containing 10% FBS, L-glutamine and P/S) and grown for 1-2 weeks to allow cells to emerge.

PCR arrays
The RNA concentration was quantified and 100 ng reverse transcribed using a RT PreAmp cDNA synthesis kit (SA Biosciences) using DNA damage signaling pathway-specific primers. Briefly, RNA was reverse transcribed and then subject to PCR amplification in the presence of primers specific for the array. The pre-amplified cDNA was mixed with SYBR Green mastermix. Arrays were performed in duplicate in a total reaction volume of 20 µl in a Rotor-Gene Q (Corbett) with the following parameters; 95°C for 10 minutes, followed by 40 two-step cycles at 95°C for 10s and 60°C for 30s. The array contained control primers for amplification of 5 housekeeping genes, to control for reverse transcription, genomic DNA contamination and PCR cycling efficiency. Arrays were analysed using SA Biosciences software (Qiagen). Microarray data have been deposited in MIAME (GSE59030, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59030).

Comet Assay
30,000 carotid or aortic human VSMCs/well or 50,000 mouse VSMCs/well were seeded in 6-well plates and left to adhere overnight at 37°C/5% CO₂. Cells were left untreated or treated with 40 µM t-BHP for 30 min, the media removed, cells washed with PBS, and media replaced. Cells were left to recover for 1-6 h (human) or 0.5-8 h (mouse) following removal of t-BHP. Cells were washed in PBS and lysates collected into PBS, centrifuged at 1000 rpm, and resuspended in PBS to a final volume of 50 µl. Following addition of 150 µl 1% low melting point agarose (LMPA) the sample was loaded on to gel-bond film, and incubated at 4°C for 10 min to allow the agarose to set. Samples were incubated overnight in lysis buffer at 4°C, washed briefly with water, and placed for 30 min in alkaline electrophoresis buffer followed by electrophoresis at 26V for 30 min at 4°C. Samples were removed, incubated in neutralization buffer (100 mM Tris pH 7.6) and subsequently submersed in ice-cold ethanol and allowed to air-dry. Samples were rehydrated following incubation in H₂O and stained with EtBr solution. Multiple images per slide were analysed using a Zeiss microscope and comet tail length/moment measured using Comet Assay IV software (Perception Instruments).

Immunocytochemistry
Identification of nuclear foci of γ-H2AX and P-ATM was performed as previously described. Briefly, VSMCs were seeded at 5000 cells per well in chamber slides and allowed to adhere overnight. Cells were treated with 40 µM t-BHP for 30 minutes, following removal cells were washed and allowed to recover. Untreated cells served as the control. Cells were washed and fixed with ice-cold MeOH for 10 minutes on ice, blocked with SignalStain antibody diluent for 1 hour at room temperature (RT), followed by incubation with primary antibody (p-ATM and γ-H2AX) at 1:100 for 1 hour at RT. Slides were washed with PBS-0.01% Tween20 and incubated with secondary antibody at 1:500 (Alexa Fluor 488 and AlexaFluor 594; Life Technologies) for 1 hour at RT in the dark. Slides were washed, mounted with DAPI and imaged. Fluorescence intensity was analysed using Image J software.
Generation and characterisation of transgenic mice

Transgene expression was determined by isolation of liver, gut and aorta from transgenic mice and negative control littermates. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer’s instructions. RNA was quantified and further purified using RQ1 RNase-Free DNase (Promega), reverse transcribed using Superscript II (Invitrogen), diluted 1:2 and subject to an initial round of PCR using the genotyping cycling conditions (described above) using primers specific for SM22α (F: 5’ GGAAGCTGTTGGAGTGAGTG3’) and NBS1 (R: 5’ TGGGCCCTGAGGATACAGT3’). 5 µl of this template was then further amplified in a second round of PCR using a second primer set specific for NBS1 (F: 5’ CGAAGTTAATCGGAGGA 3’ and R: 5’ GCCGTCTGACAGATCACA 3’). GAPDH was used as an input control and purified RNA (no RT) to exclude genomic DNA contamination. Mouse VSMCs isolated from aortas were examined using the same nested PCR conditions. PCR products were analysed on a 2% agarose gel containing EtBr.

Genotyping

Ear notches from transgenic mice were incubated with Chelex/Proteinase K for 3 hours at 56°C. Following heat inactivation at 95°C, 2 µl DNA was used in the subsequent PCR reaction. NBS1(ΔC) gene expression was analysed using forward: 5’ GGAGCCTGTGGAGTGAGTG 3’ and reverse 5’ GCATTATTATATGTTTGGTTCA 3’ primers designed to amplify a 300 bp region of NBS fused to the C-terminal HA tag using the following cycling conditions: 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 1 min. ApoE<sup>–/–</sup> genotyping was conducted according Jackson Labs (Ma, USA) protocols and cycling conditions: 94°C for 1 min, followed by 40 cycles of 94°C for 45s, 63°C for 1 min for 45s and 72°C for 90s.

SM22α-NBS1 and SM22α-(ΔC)NBS1-positive mice were backcrossed with ApoE<sup>–/–</sup> (C57BL6 background) for 5 crosses to establish a colony. For heterozygosity genotyping digested ear notches were diluted 1:10 and subject to a standard two-step qPCR (Rotor-gene Q). Briefly, 3µl DNA was used in a qPCR reaction containing 2x PCR mastermix (Life Technologies), 20x human NBS1 or mouse GAPDH Taqman primer-probes (Life Technologies) and H<sub>2</sub>O. Samples were analysed for NBS1 gene expression and normalised to GAPDH expression. Heterozygosity was defined by analysing the ratio between NBS1 and GAPDH whereby no expression corresponded to null animals; heterozygous animals were assessed as those with gene expression at 50% of the value of homozygous-positive animals.

Analysis of DNA Damage Mediators – Western blotting

Control, NBS1 and NBS(ΔC) VSMCs were seeded at 2x10<sup>6</sup> per well in 6-well plates and left to adhere overnight. Cells were treated with 40µM t-BHP for 30 minutes. t-BHP was removed and cells isolated either immediately or after 30 or 120 minutes. VSMCs were washed in ice-cold PBS, pelleted by centrifugation and lysed in IP buffer (50mM Tris pH 7.5-8, 150mM NaCl, 1% Triton, 1mM EDTA, 50mM beta-mercaptoethanol). Samples were incubated on ice for 30 minutes and supernatants collected by centrifugation at 13k for 10 min at 4°C. Protein was quantified using Bradford Assay and equal amounts of protein loaded on to a 7.5% polyacrylamide gel and proteins separated by electrophoresis. Proteins were transferred to PVDF membranes, blocked for 1 h in 5% non-fat milk or 5% BSA for phosphorylated proteins and probed. Antibodies used were against γ-H2AX (NEB #2577), total H2AX (ab11175), P-NBS1 (NEB #3001), NBS1 (ab47386), P-MRE11 (Sigma SAB4504310), MRE11 (NEB #4895), p-ATM/ATR (NEB #2851), Ser15 p53 (NEB #9284), total p53 (BD Biosciences #554157), and p21 (BD Pharmingen #556430).
Assays were performed on multiple cell isolates, in three separate experiments per isolate.

Immunoprecipitation
Control, NBS1 and NBS(ΔC) VSMCs were cultured to 90% confluence. Cells were washed with ice-cold PBS containing protease and phosphatase inhibitors, centrifuged and lysed with ice-cold RIPA buffer. Protein concentration was quantified using the BCA Assay (Pierce). 200µg protein per sample was precleared by incubating with 10µl Dynabeads Protein G (Novex #10040), the lysate was isolated and incubated with total ATM antibody (Abcam #ab78) overnight at 4˚C. 25 µl Dynabeads were added and incubated with rotary agitation for 1 hour at 4˚C. Beads were isolated and subsequently washed three times with lysis buffer. Protein was eluted in 2x sample buffer by boiling at 95˚C and subject to SDS PAGE followed by western blotting with an antibody to total NBS1 (Sigma #N3162). The total fractions were analysed for total ATM protein to serve as a control.

Mouse immunohistochemistry
Primary antibodies used for mouse immunohistochemistry, source and dilution were: p21: NEB #2946, 1: 200; γ-H2AX: #NEB 2577, 1:500; SMA: Dako M0851, 1:500 (14 mg/ml), P-ATM, Mac 3: BD 553322, 1:500 (1mg/ml), 8-oxo-G: Trevigen #4354-MC, 1:20000, PCNA: abcam #ab29, 1:6000 (0.17 mg/ml). TUNEL assay for apoptosis was performed using dUTP-digoxigenin incorporation (Roche), detection with an alkaline phosphatase-conjugated antibody to digoxigenin (Roche) and development with 5-bromo-4-chloro-3-indoyl-phosphate/p-nitroblue counterstained with 1% eosin. Images were captured using a BX51 microscope (Olympus), air-cooled CCD camera (CoolSnap) and imaging and analysis software (Soft Imaging Systems). Total number of apoptotic cells were counted per whole aortic root section and expressed as a percentage of the total number of cells.

Analysis of Atherosclerosis
Atherosclerosis extent and composition was analyzed as described previously. Briefly, sections from the aortic root and brachiocephalic arteries were stained with H&E, α-SMA, Masson’s, TUNEL, Mac3, PCNA, γ-H2AX and 8-oxo-G. Plaque extent and composition was measured using CellD analysis software. SMA content of fibrous caps was defined using Masson’s Trichrome to identify the fibrous cap region, and VSMCs in that area were counted using α-SMA on a sequential aortic root section.

Online References
Online Figure I
Heat Maps of DDR gene expression vs. multiple housekeeping genes in human aortic (n=6) or plaque (n=4) VSMCs.
### Online Table I. PCR array for DNA damage-associated genes

Mean fold change in gene expression for plaque (n=4) vs. aortic VSMCs (n=6). Green = upregulated in plaque VSMCs, Red = downregulated in plaque VSMCs.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold Change</th>
<th>Gene Symbol</th>
<th>Fold Change</th>
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Online Figure II

(A-B) Nuclear fluorescence for γ-H2AX (A) or P-ATM (B) in plaque or normal aortic VSMCs after 30 min t-BHP treatment or 30 or 120 min recovery. U=untreated.

(C) Immunocytochemistry of aortic or plaque VSMCs either untreated or after t-BHP treatment for 30 min.
Comet assay at baseline (Untreated), after t-BHP treatment for 30 min (t=0), or after t-BHP removal (1-6h recovery) in human normal aorta or plaque VSMCs.

**Online Figure III**
Online Figure IV

Immunoprecipitation of ATM and blotting with NBS1 in ApoE−/− (Ctrl), SM22α-NBS1/ApoE−/− (NBS1) or SM22α-(ΔC)NBS1/ApoE−/− VSMCs (ΔC), compared with an IgG control, demonstrating reduced association of ATM with NBS1 in SM22α-(ΔC)NBS1/ApoE−/− VSMCs. Western blot for ATM is shown below.
Online Figure V
Comet assay at baseline (Untreated), after t-BHP treatment for 30 min (t=0), or after t-BHP removal (0.5-8h recovery) in ApoE−/− (Ctrl), SM22α-NBS1/ApoE−/− (NBS1) or SM22α-ΔC/NBS1/ApoE−/− (NBS1(ΔC)) VSMCs.
Online Figure VI
Serum Lipids (A-D), body weight before (E) or after (F) fat feeding, or blood pressure (G) in control ApoE\(^{-/-}\), SM22\(\alpha\)-NBS1\(^{+/+}\)/ApoE\(^{-/-}\) (NBS1) or SM22\(\alpha\)-NBS1(ΔC)\(^{+/+}\)/ApoE\(^{-/-}\) mice (NBS1(ΔC)). n=11-15.
Online Table II.

Histological analysis of brachiocephalic plaques in Control ApoE−/−, SM22α-NBS1+−/ApoE−/− (NBS1) or SM22α-NBS1(ΔC)+−/ApoE−/− mice (NBS1(ΔC)).
Online Figure VIII. Expression of NBS1 or NBS1(ΔC) does not alter macrophage content or apoptosis

Immunohistochemistry for Mac3, γ-H2AX or TUNEL of aortic root plaques of Control ApoE<sup>−/−</sup>, SM22α-NBS1/ApoE<sup>−/−</sup> and SM22α-(ΔC)NBS1/ApoE<sup>−/−</sup> mice after 14 weeks of fat feeding. Scale bar = 500µm.
Online Table III
Plaque area and composition of aortic root plaques in Control ApoE<sup>−/−</sup> or SM22α-NBS1/ApoE<sup>−/−</sup> mice after fat feeding from 6-14w. Data are Means±SEM. n=8.

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<td>Mac3 positive area (%) Plaque)</td>
<td>6.7±1.4</td>
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Online Figure IX
DCFDA fluorescence after exposure of mouse control ApoE<sup>−/−</sup>, SM22α-NBS1<sup>+/+</sup>/ApoE<sup>−/−</sup> (NBS1) or SM22α-NBS1(ΔC)<sup>+/+</sup>/ApoE<sup>−/−</sup> VSMCs at baseline. Data are means±SEM.