ARTICLE IN PRESS

Atherosclerosis xxx (xxxx) xxx

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

DNA glycosylase Neil3 regulates vascular smooth muscle cell biology during atherosclerosis development

Ana Quiles-Jiménez [a,b,1], Ida Gregersen [a,b,1], Filip M. Segers [a,c,1], Tonje Skarpengland [a], Penelope Kroustallaki [d], Kuan Yang [a,b], Xiang Yi Kong [a,b], Knut H. Lauritzen [a], Maria B. Olsen [a], Tom Rune Karlsen [a,b], Tuula A. Nyman [e], Ellen L. Sagen [a], Vigdis Bjerkeli [a], Rajikala Suganthan [f], Ståle Nygård [g], Katja Scheffler [h], Jurriën Prins [i,j], Eric Van der Veer [i,j], Jonas DS. Øgaard [a], Yngvar Fløisand [k], Helle F. Jørgensen [l], Kirsten B. Holven [m,n], Erik A. Biessen [o], Hilde Nilsen [d], Tuva B. Dahl [a], Sverre Holm [a], Martin R. Bennett [l], Pål Aukrust [a,b,p], Magnar Bjørås [f,h], Bente Halvorsen [a,b,*]

[a] *Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway*
[b] *Faculty of Medicine, University of Oslo, Oslo, Norway*
[c] *Department of Pharmacology, Oslo University Hospital Rikshospitalet, Oslo, Norway*
[d] *Department of Clinical Molecular Biology, University of Oslo, Akershus University Hospital, Lørenskog, Norway*
[e] *Department of Immunology, Institute of Clinical Medicine, University of Oslo, Oslo University Hospital Rikshospitalet, Oslo, Norway*
[f] *Department of Microbiology, Oslo University Hospital Rikshospitalet, Oslo, Norway*
[g] *Bioinformatics Core Facility, Institute for Medical Informatics, Oslo University Hospital, Oslo, Norway*
[h] *Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology, Trondheim, Norway*
[i] *Einthoven Laboratory of Experimental Vascular Medicine, Leiden University Medical Center, Leiden, the Netherlands*
[j] *Department of Internal Medicine, Nephrology, Leiden University Medical Center, Leiden, the Netherlands*
[k] *Department of Hematology, Oslo University Hospital Rikshospitalet, Oslo, Norway*
[l] *Division of Cardiovascular Medicine, Department of Medicine, University of Cambridge, Addenbrooke's Centre for Clinical Investigation, Addenbrooke's Hospital, Cambridge, United Kingdom*
[m] *Norwegian National Advisory Unit on Familial Hypercholesterolemia, Department of Endocrinology, Morbid Obesity and Preventive Medicine, Oslo University Hospital, Oslo, Norway*
[n] *Faculty of Medicine, Department of Nutrition, Institute of Basic Medical Sciences, University of Oslo, Oslo, Norway*
[o] *Department of Pathology, University of Maastricht, Maastricht, the Netherlands*
[p] *Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital Rikshospitalet, Oslo, Norway*

ARTICLE INFO

Keywords:
Atherosclerosis
NEIL3
DNA damage repair
Vascular smooth muscle cells
Phenotypic transdifferentiation
Akt signaling

ABSTRACT

Background and aims: Atherogenesis involves a complex interaction between immune cells and lipids, processes greatly influenced by the vascular smooth muscle cell (VSMC) phenotype. The DNA glycosylase NEIL3 has previously been shown to have a role in atherogenesis, though whether this is due to its ability to repair DNA damage or to other non-canonical functions is not yet clear. Hereby, we investigate the role of NEIL3 in atherogenesis, specifically in VSMC phenotypic modulation, which is critical in plaque formation and stability.
Methods: Chow diet-fed atherosclerosis-prone *Apoe*$^{-/-}$ mice deficient in *Neil3*, and NEIL3-abrogated human primary aortic VSMCs were characterized by qPCR, and immunohistochemical and enzymatic-based assays; moreover, single-cell RNA sequencing, mRNA sequencing, and proteomics were used to map the molecular effects of Neil3/NEIL3 deficiency in the aortic VSMC phenotype. Furthermore, BrdU-based proliferation assays and Western blot were performed to elucidate the involvement of the Akt signaling pathway in the transdifferentiation of aortic VSMCs lacking Neil3/NEIL3.
Results: We show that Neil3 deficiency increases atherosclerotic plaque development without affecting systemic lipids. This observation was associated with a shift in VSMC phenotype towards a proliferating, lipid-accumulating and secretory macrophage-like cell phenotype, without changes in DNA damage. VSMC

* Corresponding author. Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Sognsvannsveien 20, 0372, Oslo, Norway.
E-mail address: Bente.Halvorsen@rr-research.no (B. Halvorsen).
[1] The authors share first co-authorship.

https://doi.org/10.1016/j.atherosclerosis.2021.02.023
Received 24 July 2020; Received in revised form 12 February 2021; Accepted 19 February 2021
Available online 23 February 2021

transdifferentiation in Neil3-deficient mice encompassed increased activity of the Akt signaling pathway, supported by cell experiments showing Akt-dependent proliferation in NEIL3-abrogated human primary aortic VSMCs.

Conclusions: Our findings show that Neil3 deficiency promotes atherosclerosis development through non-canonical mechanisms affecting VSMC phenotype involving activation of the Akt signaling pathway.

1. Introduction

Despite great improvement in the treatment of heart and stroke disease, atherosclerosis remains the leading cause of death and loss of productive life years worldwide [1]. Common risk factors such as smoking, hyperlipidemia and obesity [2] trigger sustained injury to both circulating and vascular cells that not only initiate but also accelerate the atherogenic process, with inflammation and immune activation as common mediators. Classical immune cells are of major importance in this process, but also vascular smooth muscle cells (VSMCs) have crucial anti- and pro-atherogenic functions. Thus, whereas VSMCs can induce plaque stabilization by collagen production, resulting in the formation of a fibrous cap [3], their remarkable plasticity can also induce a plaque destabilization phenotype in this cells by promoting secretion of matrix-degrading enzymes [4]. VSMC phenotypic switch is therefore an important event in the development and fate of atherosclerotic plaques, and factors regulating this process are important to identify to better understand the complex molecular mechanisms underlying atherogenesis, and thereby uncover new targets for therapy in atherosclerotic disorders.

Increased oxidative stress is a hallmark feature of atherosclerotic plaques and results in the production of reactive oxygen species (ROS) that induce nuclear and mitochondrial DNA damage. VSMCs within human atherosclerotic plaques have been shown to harbor increased DNA damage which, if left unresolved, could lead to cellular injury and apoptosis promoting plaque instability [5,6]. The base-excision repair (BER) pathway is the main repair mechanism for oxidation-induced DNA damage, initiated by lesion-specific DNA glycosylases which remove the damaged base [7]. Recent studies, however, suggest that some DNA glycosylases (e.g., NEIL3) may have functions beyond canonical DNA repair. We have recently reported increased proliferation of myofibroblasts in ruptured hearts in a *Neil3*-deficient myocardial infarction (MI)-mouse model [8], and NEIL3 expression profile is upregulated in early S phase in several human cell lines, supporting a role for NEIL3 in the regulation of cell cycle and proliferation [9]. We have also shown that a genetic variant of human *NEIL3* is associated with increased risk of MI [10], suggesting that NEIL3 is important in the development of atherosclerotic disease. Moreover, we have previously showed that Neil3 deficiency increased susceptibility to develop atherosclerosis in *Apoe*$^{-/-}$ mice fed a high fat diet involving altered function of macrophages [11]. The role of NEIL3 in the regulation of VSMC proliferation is, however, not known. In the present study, we elucidated the function of Neil3 in the development of atherosclerosis and explored the role of Neil3/NEIL3 in VSMC proliferation and phenotypic switch in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice exposed to a chow diet.

2. Materials and methods

For an extended version of Materials and Methods, please see the Supplementary Materials.

2.1. Mice

Neil3-deficient mice were generated by germline deletion of exons 3-5 as previously described [12], and backcrossed into C57BL/6J mice for 10 generations. *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice were generated by crossing *Neil3*$^{-/-}$ mice with *Apoe*$^{-/-}$ (C57BL/6J background) mice, obtained from Taconic (Denmark) for 3 generations. The *Apoe*$^{-/-}$ mice used as controls in the study were bred out from the *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ line. All mice included in the study were born at the Centre for Comparative Medicine, Oslo University Hospital Rikshospitalet, in Oslo (Norway). Genotyping of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice was carried out by use of the HotSHOT method for DNA preparation. DNA was mixed with specific primers (primer sequences can be provided upon request), PCR MasterMix (Promega, Madison, WI) and H_2O and amplified using the GeneAmp 9700 PCR System (Applied Biosystems, CA). Mice were fed a chow standard diet (RM3-P product code 801700) from Special Diets Services (www.sdsdiets.com) *ad libitum* until harvest at 26 weeks of age. All murine studies were conducted in accordance with the regulations on the use of experimental animals established by the Norwegian governmental agency "Mattilsynet" (The Norwegian Food Safety Authority), with "Forsøksdyrutvalgets tilsyn-og søknadssystem" (FOTS) application numbers 5263 and 8648.

2.2. Generation of NEIL3 knock-down primary human aortic smooth muscle cells and cell experiments

For *in vitro* experiments, we designed synthetic single-stranded RNA-DNA hybrid antisense oligonucleotides (GapmeRs) for post-transcriptional silencing of *NEIL3* messenger RNA (mRNA), as described elsewhere [13]. NEIL3 GapmeRs were designed towards exon 4 of *NEIL3* (NEIL3 KD) with sequence 5′-CUUAUUCTCTGTTGG CUUUCCA-3′, and a scrambled GapmeR (Control) used as control, with sequence 5′-UAUAUCCGGTTCTTTCUUGCUC-3' (Eurogentec, Liége, Belgium). Primary human aortic smooth muscle cells (HaoVSMCs; PromoCell GmbH, Heidelberg, Germany) were grown in PromoCell Growth Medium (PromoCell GmbH, Heidelberg, Germany) and 1% penicillin streptomycin at 37 °C with 5% CO_2. HaoVSMCs were treated with 0.5 μM NEIL3 GapmeRs for 48 h, and used for subsequent experiments on VSMC function. GapmeR-treated HaoVSMCs proliferation was assessed by means of the chemiluminescent immunoassay Cell Proliferation ELISA, BrdU (Roche Applied Science, Germany), based on the measurement of BrdU incorporation during DNA synthesis in replicating cells according to manufacturer's guidelines. The chemiluminescent signal was quantified by measuring the photons using a microplate luminometer with photomultiplier technology. These relative light units per second (rlu/s) directly correlate to the amount of DNA synthesis, hence to the number of proliferating cells in the corresponding cell microculture. For the cellular lipid-staining, HaoVSMCs were cultured as described previously, and plated out at a cell density of 2-5x10^4 cells/well in a cell culture plate, 24-well, polystyrene, Cell+ growth surface for sensitive adherent cells (Sarstedt, Oslo, Norway). *NEIL3* gene expression was knocked down using 0.5 μM GapmeRs supplied to the cellular medium. Cells without GapmeRs and with the scrambled control (0.5 μM) were used as control conditions. After 48 h pre-incubation with GapmeR solution, Dil-labeled medium oxLDL (1 μg/mL; Kalen Biomedical) was added to the culture medium. After 24 h of lipid loading, cells were extensively washed with 2x PBS to remove unspecific-bound OxLDL, and fixated using 4% PFA (Histolab). Cells were counterstained and embedded using a SlowFade Gold Antifade mountant containing DAPI nuclear stain (ThermoFisher) or Hoescht 33258 (ThermoScientific) for nuclear staining. Images were captured by use of a Nikon DS Fi1 camera on a Nikon Eclipse E400 microscope. Furthermore, scramble- and NEIL3 GapmeR-treated HaoVSMCs were incubated with 20 μg/mL oxLDL for 24 h. Cells were washed in ice-cold PBS and fixated in formaldehyde (2% in PBS) for 30 min at room

temperature. Neutral lipids were extracted twice in hexane-isopropanol 3:2 (v/v). The solvent was pooled from each extraction and evaporated under N_2, and finally solubilized in isopropanol before total cholesterol and triacylglycerol levels were analyzed (using same assays as described for murine serum samples). For experiments on the role of AKT signaling in HaoVSMCs, NEIL3 GampeR-treated HaoVSMCs were incubated for 6 h before addition of 10 μM AKT 1/2 inhibitor (Tocris, Biotechne, Minneapolis, MN) and incubated further for 18 h before harvest.

2.3. Gene expression analyses of murine whole aorta and aortic VSMCs, and HaoVSMCs

Total RNA was isolated from murine whole aorta and aortic VSMCs, and HaoVSMCs using RNeasy spin columns (Qiagen, Germany). The RNA was treated with DNase (Qiagen) and stored at −80 °C until analysis. RNA concentrations and purity were assessed by spectrophotometer absorbance (NanoDrop ND-1000 Thermo Scientific, Wilmington, DE). Synthesis of cDNA was performed using q-Script cDNA Synthesis kit (Quanta Bioscience, Gaithersburg, MD). Real-time mRNA quantification was performed using Perfecta SYBRGreen Fastmix ROX (Quanta Bioscience, Gaithersburg, MD) and the 9700HT Fast Real-Time PCR System with the proprietary SDS 2.4 software (Applied Biosystems). The RT-qPCR program was set as specified by the supplier. All primer sequences can be provided upon request. For each transcript, RT-qPCR was conducted in duplicates, and target transcript levels were quantified by the comparative Ct method using mouse or human *β-ACTIN or GAPDH* as endogenous control. *AKT1* mRNA was detected by using TaqMan assay primers and probes mix (HS00178289_m1 human AKT1, and Mm01331626_m1 Mouse Akt1; Applied Biosystems, USA). Total RNA was also used for mRNA-seq of both murine VSMCs (n = 2-3) and human HaoVSMCs (n = 2-3) performed by Novogene Co., Ltd (China). mRNA-seq raw files were trimmed with fastp (v0.20.0) [14] in paired-end mode with default setting to remove adapters and low quality reads. Filtered reads were mapped to mouse cDNA (GRCm38.p6, NCBI *Mus musculus* Annotation Release 108) and human cDNA (GRCh38.p13, NCBI *Homo sapiens* Updated Annotation Release 109.20190905) respectively [15] using Kallisto (v0.46.0) with 100 bootstrap iterations [16]. Sleuth (v0.30.0) was used to obtain deferentially expressed transcripts with Wald test, with beta values being the effect size on the natural-log transformed data, an estimator of the fold change [17]. Both RNA-seqs have been deposited in the NCBI GEO repository [18], with project number GSE140791. Pathway and Process enrichment of Gene Ontology Biological Processes analyses were performed using Metascape using default settings [19]. To assess expression of Neil3 in individual cells, we analyzed single-cell RNA sequencing (scRNA-seq) data from healthy whole aorta and lineage-traced VSMCs in plaques of *Apoe*$^{-/-}$ mice as previously described [20].

2.4. Label-free quantitative proteome analysis

Four biological replicates were used for total proteome analysis of human aortic VSMCs. Cells were treated with NEIL3 GapmeR (GM), scrambled GapmeR (SRC) or left untreated (Ctrl). Cell pellets were lysed with ProteaseMAX™ Surfactant (Promega), and proteins were reduced, alkylated and digested into peptides with trypsin (Promega). The resulting peptides were desalted and concentrated before mass spectrometry by the STAGE-TIP method using a C18 resin disk (3 M Empore). Each peptide mixture was analyzed by a nEASY-LC coupled to QExactive (Thermo Electron, Bremen, Germany) with EASY Spray PepMap® RSLC column (C18, 2 μl, 100 Å, 75 μm × 250 mm) using 120 min LC separation gradient. The resulting MS raw files were submitted to the MaxQuant software version 1.6.1.0 for protein identification and label-free quantification (LFQ). Carbamidomethyl (C) was set as a fixed modification and acetyl (protein N-term), carbamyl (N-term) and oxidation (M) were set as variable modifications. First search peptide tolerance of 20 ppm and main search error 4.5 ppm were used. Trypsin without proline restriction enzyme option was used, with two allowed miscleavages. The minimal unique + razor peptides number was set to 1, and the allowed FDR was 0.01 (1%) for peptide and protein identification. Label-free quantitation was employed with default settings. The UniProt database with 'human' entries (October 2017) was used for the database searches. Known contaminants as provided by MaxQuant and identified in the samples were excluded from further analysis. For pathway analysis, Reactome v75 database using gene set analysis PADOG (Pathway Analysis with Down-weighting of Overlapping Genes) algorithm was performed. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository [21], with dataset identifier PXD016345.

2.5. Western immunoblots of murine aorta and VSMCs

Total protein was extracted from *ex vivo* aortic VSMC outgrowth, whole murine aortas and NEIL3-abrogated HaoVSMCs by M-PER™ Mammalian Protein Extraction Reagent or T-PER™ Tissue Protein Extraction Reagent containing Halt™ Protease and Phosphatase Inhibitor, and protein concentrations were determined by Pierce™ BCA Protein Assay Kit (all from Thermo Fisher Scientific). Proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The following antibodies were used against: Tagln (ab14106, Abcam), Myocd (MAB4028, R&D systems), Akt (9272, Cell Signaling), Phospho-Akt (Thr308; 9275, Cell Signaling), GAPDH (sc-365062, Santa Cruz Biotechnology), β-Actin (A5441, Sigma-Aldrich), Anti-rabbit IgG (7074, Cell Signaling) and Anti-mouse IgG (7076, Cell Signaling). The membranes were developed with Radiance Plus Substrate (Azure Biosystems), and images captured by LAS-4000 (Fujifilm) and quantified by Image Studio Lite (Ver 5.2, Li-Cor, Lincoln, NE).

2.6. Statistics

All data were analyzed using parametric, i.e., *t*-test, and non-parametric tests, i.e., Mann-Whitney *U* test, or 2-way ANOVA (with Sidak's test) for grouped data. Data are presented as mean and standard deviation (SD) unless otherwise stated. Statistical analyses for significance were performed using GraphPad Prism 8 (La Jolla, CA) and *p*-values <0.05 were considered statistically significant. The same software performed identification of statistical outliers using Grubb's test with $\alpha = 0.05$. For Gene Ontology analyses input gene list from both murine VSMCs and HaoVSMCs *p*-value cutoff was set to ≤ 0.001. For proteomics, Perseus 1.6.1.3 was used for the statistical analysis of the total proteome MaxQuant results, with $p < 0.05$ as cut-off.

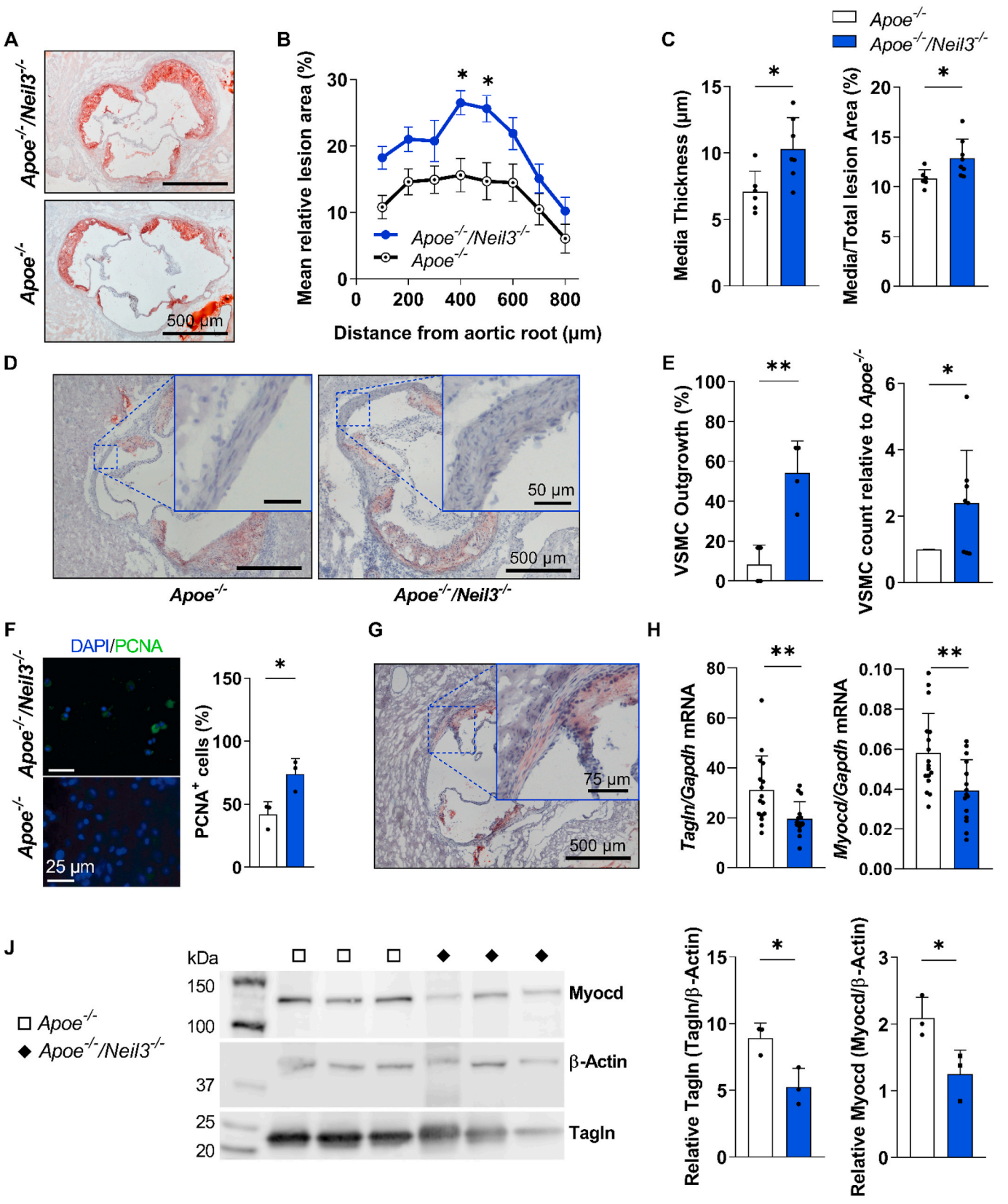
3. Results

3.1. Increased atherosclerosis in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice

Atherosclerosis was quantified by Oil Red O (ORO) staining in aortic roots of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice on chow diet at 26 weeks of age, showing significantly increased atherosclerotic lesion area in the aortic root of *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice, as compared to *Apoe*$^{-/-}$ mice (Fig. 1A and B). *En face* plaque quantification analysis with Sudan IV staining, however, revealed no significant differences in aortic arch lesions when comparing *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice (Supplementary Fig.1). Immunohistochemical staining of the aortic root using traditional cell markers showed no significant differences in total plaque composition between genotypes when analyzing macrophages (Cd68^{+}), endothelial cells (Vcam1^{+}), VSMCs (Acta2^{+}), T cells (Cd4^{+} and Cd8^{+}), or collagen composition (Sirius Red^{+}) (Supplementary Fig. 2 and 3).

3.2. Neil3 deficiency had no effects on circulating lipids or blood pressure in *Apoe*$^{-/-}$ mice fed a chow diet

Surprisingly, the increased plaque area in the aortic root of *Apoe*$^{-/}$



(caption on next page)

Fig. 1. Neil3 deficiency increases atherosclerosis with increased medial VSMC thickness and proliferation in *Apoe*-deficient mice.
(A) Representative cryosections (10 μm) of aortic roots stained with Oil Red O (ORO) and hematoxylin (original magnification 40x). (B) Atherosclerosis quantification as mean and SEM of relative lesion areas at 8 different consecutive points in the aortic root of *Apoe*$^{-/-}$ (n = 8-10) and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ (n = 8-9) mice. *$p < 0.05$ *versus Apoe*$^{-/-}$ mice (2-way ANOVA with Sidak's test). (C) Quantitative assessment of the vascular wall as medial wall thickness and percentage medial to total lesion area in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice. Media thickness measurements are an average of ≥5 measurements at different locations/valve/mouse; i.e. ≥ 15 measurements/mouse. (D) Representative pictures of ORO-stained aortic roots of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice. Magnification boxes show a representative region of interest within the aortic root, emphasizing disrupted VSMC multilayer organization in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ as compared to *Apoe*$^{-/-}$ mice. (E) VSMC outgrowth percentage and cell count from aortic explants of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice (n = 4-8). (F) Representative pictures of DAPI/PCNA co-immunostaining and quantification in aortic VSMC from outgrowth explants of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice. (G) Representative picture of ORO-staining accumulated in the medial VSMC layer. Tagln and Myocd mRNA expression (H) and protein (J) levels in thoracic aortic tissue from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice (n = 3-17). Data presented as mean and SD. T-test *$p < 0.05$, **$p < 0.01$.

$^{-/-}$/*Neil3*$^{-/-}$ mice was not accompanied by changes in serum total cholesterol, oxidized LDL, nor triglyceride levels as compared to *Apoe*$^{-/-}$ mice (Supplementary Fig. 4). Further, there were no differences in blood pressure between *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice (Supplementary Fig. 5). In addition, analysis of the cytokine profile in murine plasma presented only modest changes, with decreased levels of both pro- (i.e., Interleukin (IL)-1β, Csf3, and IL-12p40) and anti-atherogenic (i.e., IL-5) cytokines in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice (Table 1 Supplementary Materials).

3.3. Increased medial VSMC layer area and disorganization in aortic roots of *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice

Despite no changes in typical cell markers of plaque composition, ORO-stained murine aortic root specimens revealed significantly increased media layer thickness and increased percentage of media area to total aortic lesion area in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ compared to *Apoe*$^{-/-}$ mice (Fig. 1C). Interestingly, the same sections showed an increased level of VSMC disorganization inside the media layer of the aorta in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice compared to *Apoe*$^{-/-}$ controls (Fig. 1D). Increased media layer thickness, accompanied by VSMC layer disorganization in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice, point towards a role of VSMCs in our model.

3.4. Increased VSMC proliferation and phenotypic changes in aortas of *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice

Based on these findings, we further assessed VSMC proliferation in an *ex vivo* explant assay of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ aortas. Both measurement of *ex vivo* aortic VSMC outgrowth and cell count from aortic arch sections showed increased proliferation of aortic VSMCs in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice compared to *Apoe*$^{-/-}$ (Fig. 1E). Immunofluorescent staining on VSMCs from aortic explants with proliferating cell nuclear antigen (PCNA), an established marker of cell proliferation, identified increased Pcna^{+} VSMCs in aortic explants from *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ specimens compared to *Apoe*$^{-/-}$ controls (Fig. 1F). Inspection of ORO-stained sections of murine aortic roots revealed lipid accumulation inside medial aortic VSMCs in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ (Fig. 1G). In addition, mRNA expression (Fig. 1H) and protein levels (Fig. 1J) of VSMC differentiation markers transgelin (Tagln, SM22α) and myocardin (Myocd) [22] were decreased in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ aortas, indicating cell de-differentiation. These results point towards phenotypic modulation of VSMCs, presenting a proliferating, de-differentiated, and lipid-accumulating phenotype in *Apoe*-deficient mice lacking Neil3.

3.5. Neil3 is expressed in a subset of murine VSMCs in Apoe-deficient mice

To further examine the role of Neil3 in the regulation of VSMCs, we analyzed single cell-RNA sequencing (scRNA-seq) datasets generated from arteries of *Apoe*-deficient mice [20]. Though only in a small percentage of cells, *Neil3* expression was present in some VSMCs in plaques, which interestingly mostly fell within the expression domain of the progenitor cell marker *Ly6/Sca-1*, which is upregulated in VSMCs within lesions (Supplementary Fig. 6A and B). In contrast, *Neil3* was not found in any lineage of VSMCs in healthy vessels (Supplementary Fig. 6C and D). These data further support a link between Neil3 and VSMC proliferation.

3.6. NEIL3 deficiency induces increased proliferation, phenotypic transdifferentiation and lipid uptake in primary human aortic VSMCs

Our findings so far suggest that increased atherosclerosis in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice could be due to a modulation in VSMC phenotype with increased cell proliferation and lipid uptake. To further elucidate the effect of Neil3 deficiency on VSMC phenotype, we established an *in vitro* model in which *NEIL3* expression was abrogated in primary human aortic VSMCs (HaoVSMC) by means of post-transcriptional gene silencing. Expression of *NEIL3* in HaoVSMCs was significantly reduced (>70%) after NEIL3 GapmeR treatment for 18 h (Supplementary Fig. 7), and NEIL3-deficient HaoVSMCs demonstrated significantly increased proliferation compared to controls (Fig. 2). VSMC transdifferentiation to a more macrophage- or chondrocyte-like cell phenotype has been previously described to promote atherosclerosis [20,23], and notably the NEIL3-deficient HaoVSMCs showed increased mRNA levels of markers associated with both proliferation and phenotypic switching i.e., *CD68, TGF-β, PAI-1,* and *matrix metalloproteinase (MMP)-2* (Fig. 2B). Conversely, *ACTA2*, a canonical VSMC marker, did not show differences between NEIL3 abrogated-HaoVSMCs and controls. Also, NEIL3 KD-HaoVSMC displayed increased secretion of MMP-2, a major player in plaque progression and destabilization (Fig. 2C) [24], as well as increased lipid uptake compared to controls (Fig. 2D-F), suggesting a phenotypic shift also at the functional level. Taken together, these findings indicate that NEIL3 deficiency drives VSMCs towards a more macrophage-like cell phenotype, which could contribute to atherosclerotic plaque progression.

3.7. Unaltered genome integrity in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice

Since Neil3 is a DNA repair enzyme removing oxidation-induced DNA base lesions as part of the BER pathway, we investigated whether the aforementioned observations were a result of attenuated DNA repair capacity in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice. After measuring accumulation of damage in genomic DNA, we did not find differences in neither nuclear nor mitochondrial DNA integrity of murine aortic VSMCs when comparing the two genotypes (Supplementary Fig. 8A). The bulk level of the major oxidized DNA base lesion 8-oxoG displayed was further similar in the nuclear genome of *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ and *Apoe*$^{-/-}$ livers (Supplementary Fig. 8B). These results demonstrate that the effect of Neil3 on atherogenesis is probably not directly related to DNA repair deficiency.

3.8. Transcriptomic and proteomic analyses confirm phenotypic modulation in both murine and human aortic Neil3/NEIL3-deficient VSMCs

To further explore the NEIL3-related mechanisms underlying this

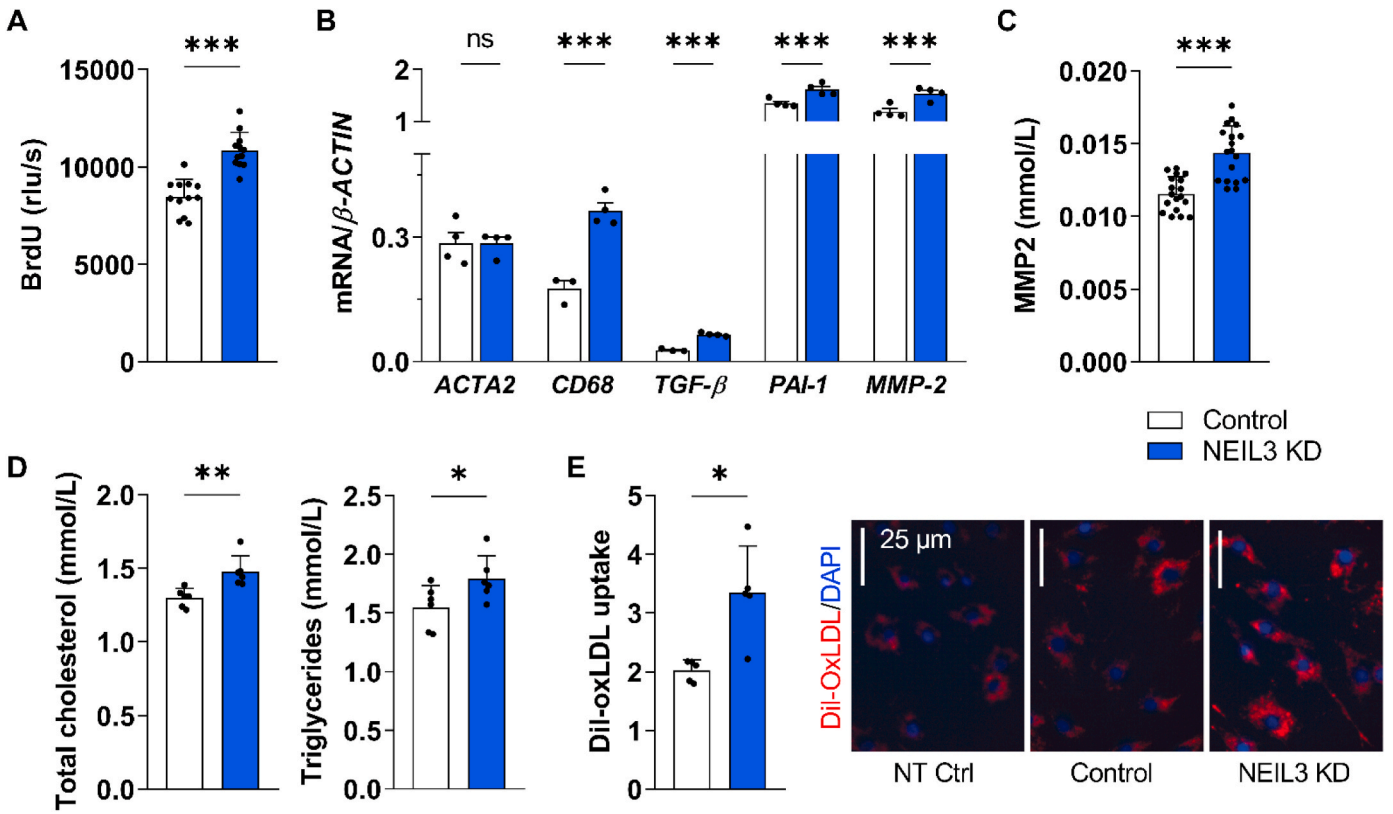


Fig. 2. NEIL3-deficient human aortic VSMC (HaoVSMC) show phenotypic modulation with increased proliferation and lipid uptake.
(A) NEIL3-deficient HaoVSMC positive for BrdU proliferation marker compared to controls (n = 5). Data presented as average from three pooled independent experiments. (B) mRNA expression levels of *ACTA2*, *CD68*, *TGFβ*, *PAI-1*, and *MMP-2* (n = 4). (C) MMP-2 secretion in NEIL3 deficient-HaoVSMC (n = 4). (D) Triglycerides and total cholesterol uptake in NEIL3 deficient-HaoVSMC (n = 6). (E) Quantification and representative pictures of lipid loading co-staining with Dil-OxLDL and DAPI in NEIL3-deficient HaoVSMC and controls. Data presented as mean and SD. T-test $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. ns, not statistically significant. KD, knock-down; NT Ctrl, non-treatment control; rlu/s, relative light units per second.

VSMC phenotype we performed different sequencing analyses. mRNA-seq revealed that aortic VSMCs from *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice were enriched in genes involved in cell proliferation and development (i.e., GO biological processes included extracellular structure organization/adhesion, mitochondrial outer layer membrane permeabilization/apoptosis, differentiation and morphogenesis, responses to growth factors and cytokines) compared to *Apoe*$^{-/-}$ mice (Fig. 3A and Supplementary Table 2). Similarly, mRNA-seq of NEIL3-deficient HaoVSMCs revealed enrichment in genes associated with cell adhesion, proliferation/apoptosis, and response to growth factors, morphogenesis, differentiation, and development (Fig. 4A and Supplementary Table 3).

Phenotypic modulation in NEIL3-deficient HaoVSMCs was further confirmed using label-free quantitative proteomics. Out of 139 significantly regulated proteins, we found 29 proteins associated with cell proliferation, migration, morphology, and structural organization (Fig. 4C and Supplementary Table 4).

3.9. Lack of Neil3/NEIL3 affects VSMC proliferation and lipid uptake through the Akt signaling pathway

The Akt signaling pathway has been extensively linked to VSMC proliferation and phenotypic switching [25,26]. Although it was not of the most up-regulated genes in NEIL3-abrogated HaoVSMC mRNA-seq, a more *in-depth* examination of the HaoVSMC proteomics by PADOG Reactome analysis showed that the top differentially upregulated pathway in HaoVSMCs lacking NEIL3 is "NF-κB activated and signals survival" (Supplementary Table 5), which has been shown to be a downstream target of the Akt signaling pathway promoting cell survival [27]. Importantly, murine Akt was one of the gene hits regulating most pathways in the mRNA-seq analysis in aortic VSMCs from *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice as compared to *Apoe*$^{-/-}$ controls (Fig. 3B and Supplementary Table 2). Quantitative PCR analysis confirmed that *Akt* mRNA levels were significantly increased in murine aortas from *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ as compared to *Apoe*$^{-/-}$ mice (Fig. 5A). We also observed increased Akt phosphorylation in western blots of explant aortic VSMCs and whole aorta from *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice as compared to *Apoe*$^{-/-}$ mice (Fig. 5B and C). In addition, the increased proliferation and lipid uptake in NEIL3-deficient HaoVSMCs was strongly reversed by addition of an AKT inhibitor (Fig. 5D). These findings combined suggest the Akt signaling pathway as a novel target for NEIL3-regulated control of VSMC functions.

4. Discussion

NEIL3/Neil3 is a DNA glycosylase initiating repair of oxidized DNA bases which functions in the maintenance of genomic stability of vertebrates [28,29]. In the present study, we show that Neil3 deficiency in *Apoe*$^{-/-}$ mice on chow diet markedly increased plaque development in the aortic root without affecting the systemic lipid and cytokine profile. We demonstrate that increased plaque development in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice was associated with a shift in VSMC phenotype towards a proliferating, lipid-accumulating and secretory macrophage-like cell phenotype. Plaque area was however not significantly different in the aortic arch, when comparing the two genotypes. The aortic root is often the first area in the aorta to develop atherosclerosis in murine models, indicating that Neil3 deficiency could be more essential in the initiation steps of atherogenesis [30]. Further, it could be that the VSMC phenotype observed in our model is most

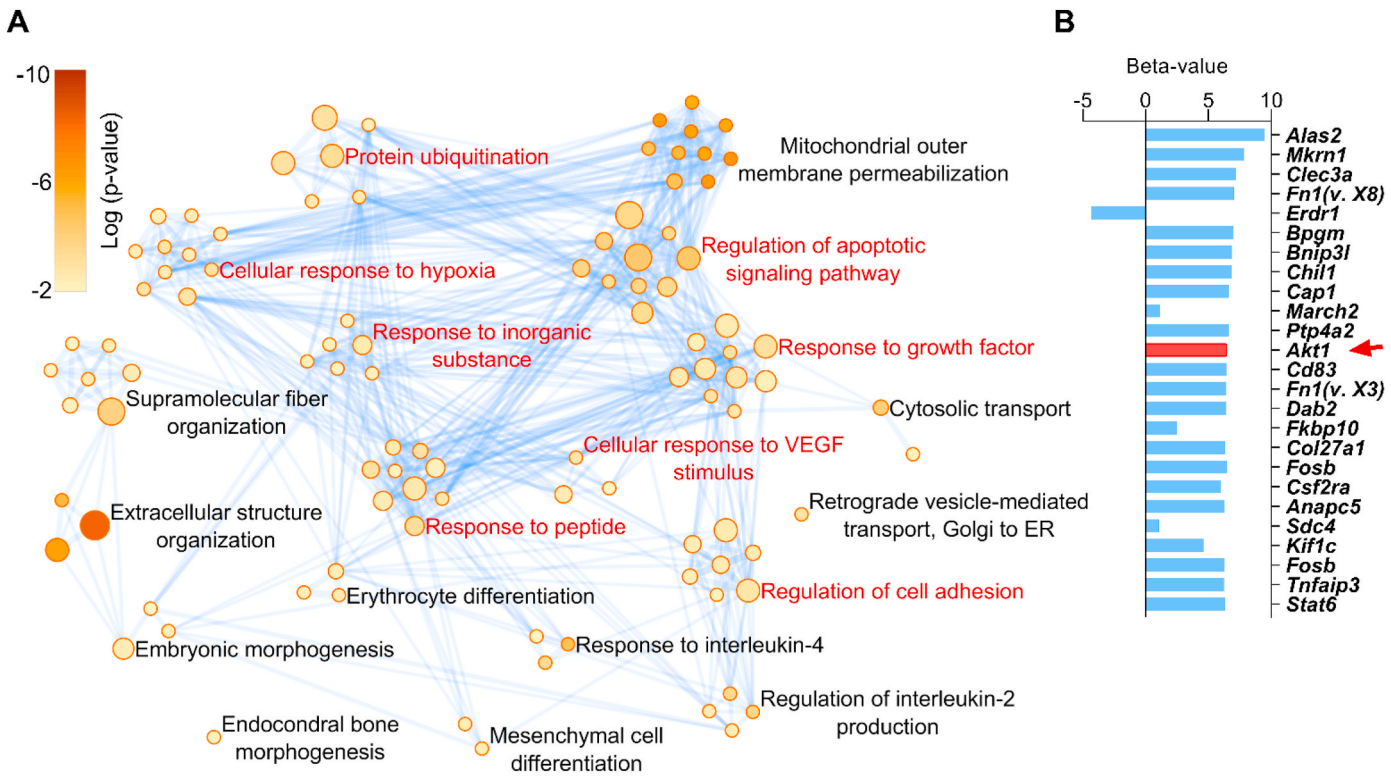


Fig. 3. RNA-sequencing analysis confirms phenotypic modulation in aortic VSMC from *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice.
(A) Enriched Gene Ontology (GO) biological processes in gene hits from RNA sequencing of aortic VSMC of *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice. GO terms in red represent *Akt1* gene hits. Clusters comprehend GO subcategories as nodes colored by *p*-value with edge thickness representing Cosine similarity between nodes. (B) Top 25 beta-values (natural-log fold change) of differentially expressed genes (DEGs), in order of statistical significance, from aortic VSMC RNA sequencing of *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ *versus Apoe*$^{-/-}$ mice. Red bar and arrow highlight *Akt1* hit. DEGs $p < 0.001$. *v.* stands for transcript version. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

important for the initiation of atherosclerosis, and not for its progression, and plaque development in the aortic arch (later stage of atherogenesis).

Several studies have pinpointed the remarkable plasticity of VSMCs in the vessel wall, where VSMCs undergo complex structural and functional changes during atherosclerosis development [31]. Herein, *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ aortic roots presented increased media thickness and media area percentage to total plaque area compared to controls, with remarkably no changes in *ACTA2*, the traditional marker for VSMCs. In fact, in the last years it has become clear that correct identification of VSMCs poses a challenge, since VSMCs can undergo phenotypic switching from an anti-atherogenic to a pro-atherogenic lesional cell phenotype where the traditional contractile VSMC markers, e.g., Acta2, are downregulated, and classical markers of macrophages, e.g., Cd68, are upregulated [31]. Indeed, we showed that knock-down of NEIL3 in human aortic VSMCs induced upregulation of *CD68, TGF*-β, *PAI-1* and *MMP-2*, but not *ACTA2*. Importantly, this phenotypic switch was also seen at the functional level as NEIL3 deficiency induced increased proliferation and lipid accumulation as well as boosted secretory capacity (i.e., MMP-2) in human aortic VSMCs. Moreover, in *Apoe*$^{-/-}$ mice we found that *Neil3* was expressed in a small number of lineage-traced plaque VSMCs within the expression domain of the progenitor marker *Ly6a/Sca-1*. Indeed, Sca1^{+} progenitor cells have been found in the adventitia proximal to atherosclerotic lesions where they differentiate into pro-atherogenic Acta2-negative VSMCs [32].

A growing number of studies support a possible distinct function of NEIL3/Neil3 beyond canonical DNA repair. Recent work has demonstrated that Neil3 is involved in the regulation of cardiac fibroblast proliferation in Neil3-deficient mice following MI [8]. Moreover, Neil3 has been shown to affect the proliferation of murine embryonic fibroblasts in an *in vitro* system [12,33]. The present findings suggest that Neil3 can also regulate VSMC proliferation and transdifferentiation, thereby affecting the susceptibility to develop atherosclerosis. Taken together, these findings raise the possibility of Neil3 being an important regulator or switch of fibrogenesis and extracellular matrix through non-canonical mechanisms. Most importantly, regulation of fibrosis is of outmost significance not only in atherosclerosis and other cardiovascular diseases, but also in several other inflammatory disorders. In our opinion, therapy targeting NEIL3 could be of interest in future studies on these disorders.

Our results show that lack of Neil3/NEIL3 promotes atherosclerosis, possibly driven by VSMC proliferation and phenotypic switching. However, the proliferative phenotype was not explained by insults to genomic DNA in *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice. To elucidate the molecular cause of our observations we performed mRNA-seq of aortic VSMCs from both murine and human models. These analyses showed that VSMCs lacking Neil3 were enriched in genes associated with extracellular structure organization/adhesion, proliferation, differentiation and development, as well as responses to growth factors, cytokines and stress stimuli. Furthermore, proteomic analysis of human aortic VSMCs lacking NEIL3 revealed regulation of proteins involved in cell proliferation, survival, and phenotypic modulation. One striking finding in our RNA-seq data analysis was the ubiquity of one of the gene hits, the RAC-alpha serine/threonine-protein kinase Akt1/PKB. In fact, Akt1 has been previously seen to influence the migratory and survival capabilities of VSMCs in the context of cardiac dysfunction and plaque stability in atherosclerosis [26]. Akt signaling has also been shown to promote phenotypic switching in aortic VSMCs from a contractile to synthetic phenotype [34]. We showed that AKT inhibition strongly counteracted proliferation in NEIL3-abrogated primary human aortic VSMCs. Our data suggest that NEIL3 deficiency promotes VSMC proliferation and potentially also atherogenesis through activation of the Akt signaling

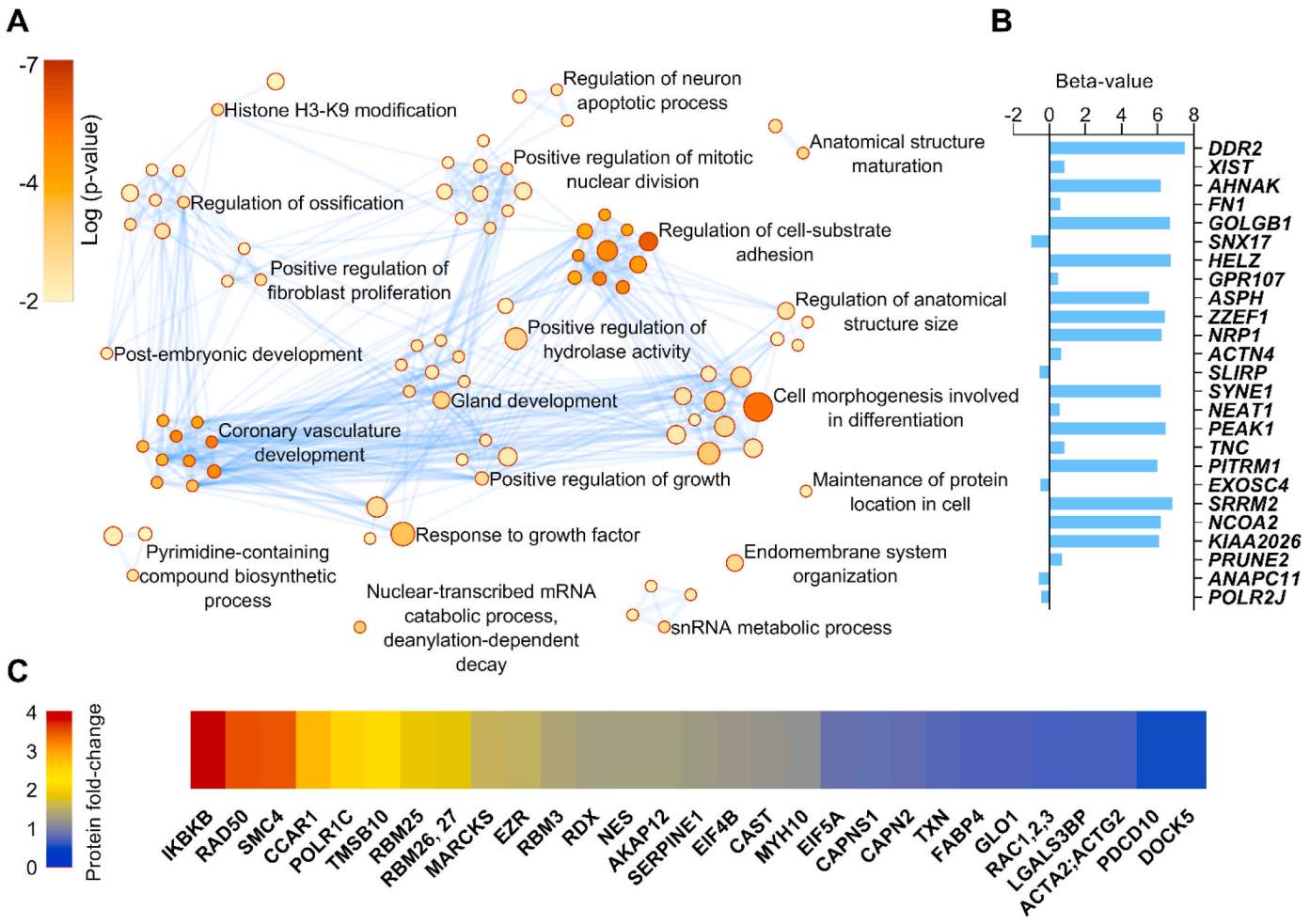


Fig. 4. High-throughput analyses confirm phenotypic modulation in NEIL3-deficient HaoVSMC.
(A) Enriched Gene Ontology (GO) biological processes in gene hits from RNA sequencing of NEIL3-deficient HaoVSMC. Clusters comprehend GO subcategories as nodes colored by *p*-value with edge thickness representing Cosine similarity between nodes. (B) Top 25 beta-values (natural-log fold change) of differentially expressed genes (DEGs), in order of statistical significance, from NEIL3-deficient *versus* control HaoVSMC RNA sequencing. DEGs $p < 0.001$. (C) Heatmap of protein fold-change involved in phenotypic modulation in NEIL3-deficient HaoVSMC.

pathway.

We have previously shown that *NEIL3* expression was increased in human carotid plaques, and that Neil3 deficiency increased susceptibility to develop atherosclerosis in *Apoe*$^{-/-}$ mice fed a high fat diet potentially involving altered macrophage function [11]. In the present study, we show that *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice fed a regular diet also developed enhanced atherosclerosis and, in contrast to high fat diet-fed mice, plaque development appears to be caused by transdifferentiated VSMCs without any alterations in circulating lipids or plaque macrophage accumulation. The reason for these diet-dependent differences on *Neil3* deficiency in *Apoe*$^{-/-}$ is at present not clear, but it could reflect the importance of metabolic stress on phenotype development, despite similar genotype.

The present study has some limitations such as the lack of quantitative analysis of lipid accumulation in VSMC *in vivo*, and the effect of Akt inhibition on a more thorough characterization of the VSMC phenotype. Moreover, our results on transdifferentiated VSMC characterization are to some extend limited due to the lack of analysis to thoroughly characterize the VSMC phenotype. VSMC plasticity is highly dependent on disease context and degree of disease [31], which makes a comprehensive understanding of their nature difficult to study. Further studies using targeted *NEIL3/Neil3* knock-out in VSMCs, as well as VSMC lineage-tracing models and extensive single cell-RNA sequencing, could uncover the precise impact of *NEIL3/Neil3* deficiency in VSMC and its role in atherosclerosis. In addition, the HaoVSMCs in this study were not a total knock-out of NEIL3, since we achieved between 70 and 80% knock-down, which has to be considered for final interpretations. Moreover, the lack of available Neil3 antibodies makes functional and targeted analysis of Neil3 regulation and function challenging, as transcription is the only assessment to address Neil3 expression to date. Finally, and most importantly, the exact molecular mechanisms by which Neil3 modulates atherogenesis and VSMC in particular are still unknown.

In summary, our results suggest that NEIL3/Neil3 is a novel player in the regulation of VSMC biology, controlling cell proliferation and differentiation, including structural and migratory functions. Lack of Neil3 in atherosclerosis-prone mice promotes a pro-atherogenic macrophage-like lesional VSMC phenotype through non-canonical mechanisms that involves regulation of the Akt signaling pathway, possibly exacerbating atherosclerosis in our model.

Financial support

This work was supported by the Norwegian Research Council RC grant [grant 144139 to B.H.] and the Norwegian Health Authority South-East [grant number 2018084 to B.H.], and the British Heart Foundation (BHF) Grants [grant numbers CH2000003/12800, RG/13/14/30314 to H.J., M.R.B.], the BHF Centre for Cardiovascular Research

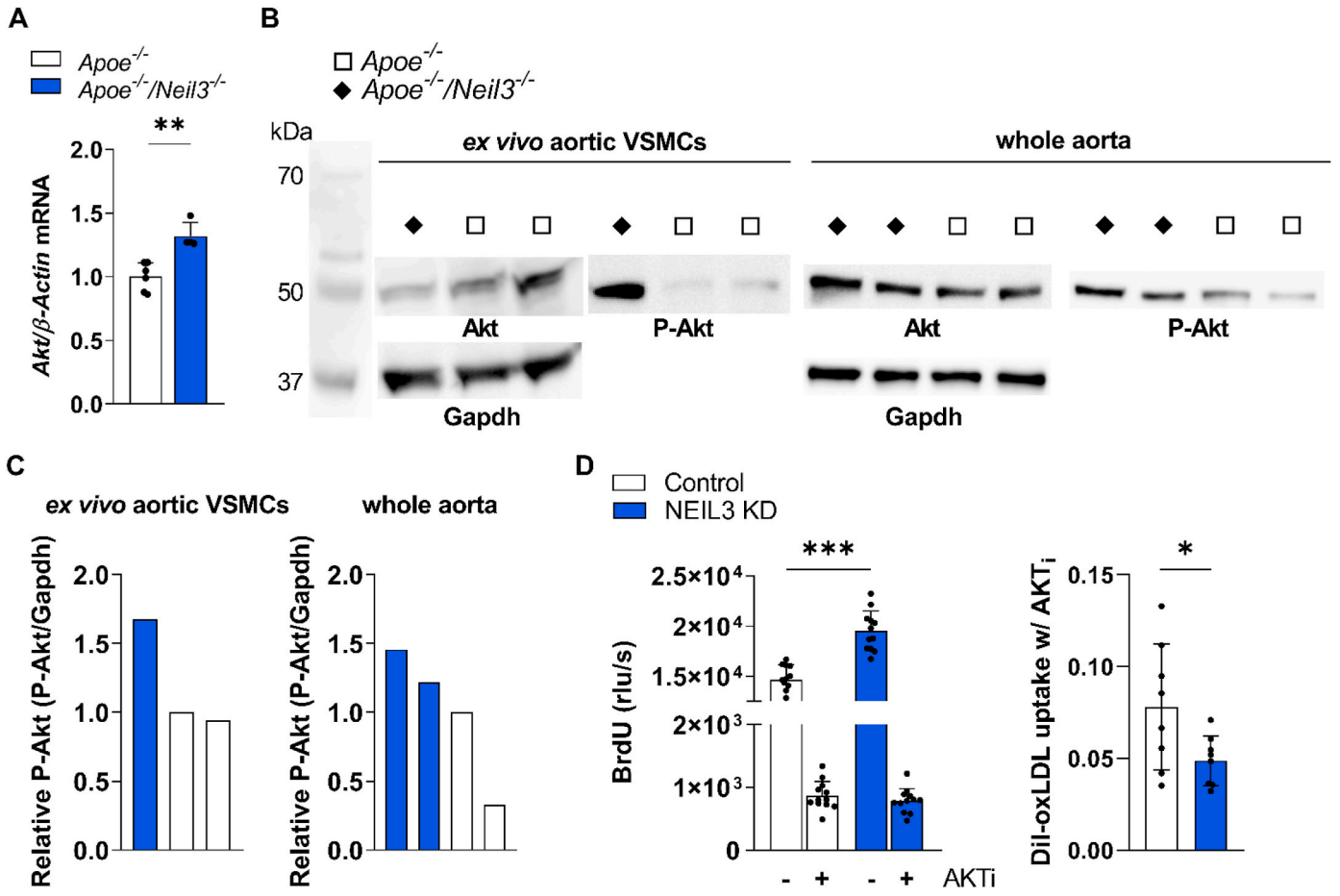


Fig. 5. Akt/AKT affects proliferation in the absence of NEIL3.
(A) *Akt* mRNA expression levels in whole aorta from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice (n = 4-6). Protein immunodetection (B) and quantification (C) of phosphorylated Akt (P-Akt) compared to non-phosphorylated Akt (Akt) and GAPDH, in *ex vivo* outgrowth aortic VSMCs and whole aorta from *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$/*Neil3*$^{-/-}$ mice. (D) Effects of AKT inhibition (AKT$_i$) on proliferation and lipid uptake in NEIL3-abrogated HaoVSMCs compared to controls (n = 8-12). T-test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. AKTi, AKT inhibitor; rlu/s, relative light units per second.

Excellence, and the National Institute for Health Research Cambridge Biomedical Research Centre to H.J., M.R.B.

CRediT authorship contribution statement

Ana Quiles-Jiménez: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Ida Gregersen:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Filip M. Segers:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Tonje Skarpengland:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Penelope Kroustallaki:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Kuan Yang:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Xiang Yi Kong:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Knut H. Lauritzen:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Maria B. Olsen:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Tom Rune Karlsen:** Validation, Investigation, Writing - review & editing. **Tuula A. Nyman:** Validation, Investigation, Writing - review & editing. **Ellen L. Sagen:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Vigdis Bjerkeli:** Validation, Investigation, Writing - review & editing. **Rajikala Suganthan:** Validation, Investigation, Writing - review & editing. **Ståle Nygård:** Formal analysis, Visualization, Writing - review & editing. **Katja Scheffler:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Jurriën Prins:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Eric Van der Veer:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Jonas DS. Øgaard:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Yngvar Fløisand:** Validation, Investigation, Writing - review & editing. **Helle F. Jørgensen:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Kirsten B. Holven:** Formal analysis, Visualization, Writing - review & editing. **Erik A. Biessen:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Hilde Nilsen:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Tuva B. Dahl:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Sverre Holm:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Martin R. Bennett:** Validation, Investigation, Formal analysis, Visualization, Writing - review & editing, Funding acquisition. **Pål Aukrust:** Conceptualization, Methodology, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Magnar Bjørås:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing - review & editing. **Bente Halvorsen:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Visualization, Writing -

original draft, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Holven reports grants and/or personal fees from Tine SA, Mills DA, Olympic Seafood, Amgen, Sanofi, Kaneka and Pronova, none of which are related to the content of this manuscript. Dr. Bennett reports grants from British Heart Foundation, grants from National Institute for Health Research Cambridge Biomedical Research Centre, during the conduct of the study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2021.02.023.

References

[1] P. Libby, P.M. Ridker, G.K. Hansson, Progress and challenges in translating the biology of atherosclerosis, Nature 473 (7347) (2011) 317–325.
[2] I. Tzoulaki, et al., Worldwide exposures to cardiovascular risk factors and associated Health effects, Circulation 133 (23) (2016) 2314–2333, https://doi.org/10.1161/CIRCULATIONAHA.115.008718.
[3] W. Insull, The pathology of atherosclerosis: plaque development and plaque responses to medical treatment, Am. J. Med. 122 (1, Supplement) (2009) S3–S14, https://doi.org/10.1016/j.amjmed.2008.10.013.
[4] A.C. Doran, N. Meller, C.A. McNamara, Role of smooth muscle cells in the initiation and early progression of atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 28 (5) (2008) 812–819, https://doi.org/10.1161/atvbaha.107.159327.
[5] T. Cervelli, et al., DNA Damage and Repair in atherosclerosis: current Insights and future perspectives, Int. J. Mol. Sci. 13 (12) (2012) 16929–16944, https://doi.org/10.3390/ijms131216929.
[6] K. Gray, et al., Effects of DNA Damage in smooth muscle Cells in atherosclerosis, Circ. Res. 116 (5) (2015) 816–826, https://doi.org/10.1161/CIRCRESAHA.116.304921.
[7] S.Z. Krokeide, et al., Human NEIL3 is mainly a monofunctional DNA glycosylase removing spiroimindiohydantoin and guanidinohydantoin, DNA Repair 12 (12) (2013) 1159–1164, https://doi.org/10.1016/j.dnarep.2013.04.026.
[8] M.B. Olsen, et al., NEIL3-Dependent Regulation of cardiac fibroblast proliferation prevents myocardial rupture, Cell Rep. 18 (1) (2017) 82–92, https://doi.org/10.1016/j.celrep.2016.12.009.
[9] C.G. Neurauter, L. Luna, M. Bjørås, Release from quiescence stimulates the expression of human NEIL3 under the control of the Ras dependent ERK-MAP kinase pathway, DNA Repair 11 (4) (2012) 401–409, https://doi.org/10.1016/j.dnarep.2012.01.007.
[10] T. Skarpengland, et al., Genetic variants in the DNA repair gene NEIL3 and the risk of myocardial infarction in a nested case-control study. The HUNT study, DNA Repair (Amst.) 28 (2015) 21–27, https://doi.org/10.1016/j.dnarep.2015.01.013.
[11] T. Skarpengland, et al., Neil3-dependent base excision repair regulates lipid metabolism and prevents atherosclerosis in Apoe-deficient mice, Sci. Rep. 6 (2016) 28337, 10.1038/srep28337 http://www.nature.com/articles/srep28337#supplementary-information.
[12] Y. Sejersted, et al., Endonuclease VIII-like 3 (Neil3) DNA glycosylase promotes neurogenesis induced by hypoxia-ischemia, Proc. Natl. Acad. Sci. U. S. A. 108 (46) (2011) 18802–18807, https://doi.org/10.1073/pnas.1106880108.
[13] A. Aartsma-Rus, Overview on AON design, in: A. Aartsma-Rus (Ed.), Exon Skipping: Methods and Protocols, Humana Press, Totowa, NJ, 2012, pp. 117–129.
[14] S. Chen, et al., fastp: an ultra-fast all-in-one FASTQ preprocessor, Bioinformatics 34 (17) (2018) i884–i890, https://doi.org/10.1093/bioinformatics/bty560.
[15] N.A. O'Leary, et al., Reference sequence (RefSeq) database at NCBI: current status, taxonomic expansion, and functional annotation, Nucleic Acids Res. 44 (D1) (2015) D733–D745, https://doi.org/10.1093/nar/gkv1189.
[16] N.L. Bray, et al., Near-optimal probabilistic RNA-seq quantification, Nat. Biotechnol. 34 (5) (2016) 525–527, https://doi.org/10.1038/nbt.3519.
[17] H. Pimentel, et al., Differential analysis of RNA-seq incorporating quantification uncertainty, Nat. Methods 14 (7) (2017) 687–690, https://doi.org/10.1038/nmeth.4324.
[18] T. Barrett, et al., NCBI GEO: archive for functional genomics data sets—update, Nucleic Acids Res. 41 (D1) (2012) D991–D995, https://doi.org/10.1093/nar/gks1193.
[19] Y. Zhou, et al., Metascape provides a biologist-oriented resource for the analysis of systems-level datasets, Nat. Commun. 10 (1) (2019) 1523, https://doi.org/10.1038/s41467-019-09234-6.
[20] L. Dobnikar, et al., Disease-relevant transcriptional signatures identified in individual smooth muscle cells from healthy mouse vessels, Nat. Commun. 9 (1) (2018) 4567, https://doi.org/10.1038/s41467-018-06891-x.
[21] Y. Perez-Riverol, et al., The PRIDE database and related tools and resources in 2019: improving support for quantification data, Nucleic Acids Res. 47 (D1) (2019) D442–D450, https://doi.org/10.1093/nar/gky1106.
[22] M.W. Huff, J.G. Pickering, Can a vascular smooth muscle-derived foam-cell really change its spots? Arterioscler. Thromb. Vasc. Biol. 35 (3) (2015) 492–495, https://doi.org/10.1161/ATVBAHA.115.305225.
[23] M.R. Bennett, S. Sinha, G.K. Owens, Vascular smooth muscle cells in atherosclerosis, Circ. Res. 118 (4) (2016) 692–702, https://doi.org/10.1161/circresaha.115.306361.
[24] S. Galis Zorina, J. Khatri Jaikirshan, Matrix metalloproteinases in vascular remodeling and atherogenesis, Circ. Res. 90 (3) (2002) 251–262, https://doi.org/10.1161/res.90.3.251.
[25] E. Stabile, et al., Akt controls vascular smooth muscle cell proliferation in vitro and in vivo by delaying G1/S exit, Circ. Res. 93 (11) (2003) 1059–1065, https://doi.org/10.1161/01.Res.0000105086.31909.1b.
[26] C. Fernandez-Hernando, et al., Absence of Akt1 reduces vascular smooth muscle cell migration and survival and induces features of plaque vulnerability and cardiac dysfunction during atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 29 (12) (2009) 2033–2040, https://doi.org/10.1161/atvbaha.109.196394.
[27] J.A. Romashkova, S.S. Makarov, NF-κB is a target of AKT in anti-apoptotic PDGF signalling, Nature 401 (6748) (1999) 86–90, https://doi.org/10.1038/43474.
[28] M. Liu, et al., The mouse ortholog of NEIL3 is a functional DNA glycosylase in vitro and in vivo, Proc. Natl. Acad. Sci. Unit. States Am. 107 (11) (2010) 4925–4930, https://doi.org/10.1073/pnas.0908307107.
[29] J. Zhou, et al., NEIL3 repairs telomere Damage during S Phase to secure chromosome Segregation at mitosis, Cell Rep. 20 (9) (2017) 2044–2056, https://doi.org/10.1016/j.celrep.2017.08.020.
[30] P.A. VanderLaan, C.A. Reardon, G.S. Getz, Site specificity of atherosclerosis, Arterioscler. Thromb. Vasc. Biol. 24 (1) (2004) 12–22, https://doi.org/10.1161/01.ATV.0000105054.43931.f0.
[31] S. Allahverdian, et al., Smooth muscle cell fate and plasticity in atherosclerosis, Cardiovasc. Res. 114 (4) (2018) 540–550, https://doi.org/10.1093/cvr/cvy022.
[32] Y. Hu, et al., Abundant progenitor cells in the adventitia contribute to atherosclerosis of vein grafts in ApoE-deficient mice, J. Clin. Invest. 113 (9) (2004) 1258–1265, https://doi.org/10.1172/jci19628.
[33] V. Rolseth, et al., Loss of Neil3, the major DNA glycosylase activity for removal of hydantoins in single stranded DNA, reduces cellular proliferation and sensitizes cells to genotoxic stress, Biochim. Biophys. Acta 1833 (5) (2013) 1157–1164, https://doi.org/10.1016/j.bbamcr.2012.12.024.
[34] S.B. Zhu, et al., TGF-beta1 induces human aortic vascular smooth muscle cell phenotype switch through PI3K/AKT/ID2 signaling, Am. J. Transl. Res. 7 (12) (2015) 2764–2774.