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Atherosclerosis-associated differentially methylated regions can reflect the disease phenotype and are often at enhancers

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HIGHLIGHTS

- Preferential gain of atherosclerosis-linked DNA methylation at aorta enhancers.
- Hypermethylation at enhancers in some atherosclerosis-downregulated genes.
- Atherosclerosis-linked hyper- and hypomethylation have different functional associations.
- Smooth muscle cell phenotype changes explain their leukocyte-like DNA methylation.
- Important to consider enhancer epigenetic as well as promoter changes in disease.

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ABSTRACT

Background and aims: Atherosclerosis is a widespread and complicated disease involving phenotypic modulation and transdifferentiation of vascular smooth muscle cells (SMCs), the predominant cells in aorta, as well as changes in endothelial cells and infiltrating monocytes. Alterations in DNA methylation are likely to play central roles in these phenotypic changes, just as they do in normal differentiation and cancer.
Methods: We examined genome-wide DNA methylation changes in atherosclerotic aorta using more stringent criteria for differentially methylated regions (DMRs) than in previous studies and compared these DMRs to tissue-specific epigenetic features.
Results: We found that disease-linked hypermethylated DMRs account for 85% of the total atherosclerosis-associated DMRs and often overlap aorta-associated enhancer chromatin. These hypermethylated DMRs were associated with functionally different sets of genes compared to atherosclerosis-linked hypomethylated DMRs. The extent and nature of the DMRs could not be explained as direct effects of monocyte/macrophage infiltration. Among the known atherosclerosis- and contractile SMC-related genes that exhibited hypermethylated DMRs at aorta enhancer chromatin were *ACTA2* (aorta α2 smooth muscle actin), *ELN* (elastin), *MYOCD* (myocardin), *C9orf3* (miR-23b and miR-27b host gene), and *MYH11* (smooth muscle myosin). Our analyses also suggest a role in atherosclerosis for developmental transcription factor genes having little or no previous association with atherosclerosis, such as *NR2F2* (*COUP-TFII*) and *TBX18*.
Conclusions: We provide evidence for atherosclerosis-linked DNA methylation changes in aorta SMCs that might help minimize or reverse the standard contractile character of many of these cells by down-modulating aorta SMC-related enhancers, thereby facilitating pro-atherosclerotic phenotypic changes in many SMCs.

1. Introduction

Smooth muscle cells (SMCs), the predominant cells in aorta, can display remarkable phenotypic plasticity, as seen in atherosclerosis. Atherosclerosis involves initial damage to the single-cell endothelial layer of the intima, abnormal retention of lipids, a chronic inflammatory response, and vascular remodeling entailing changes in SMCs located in the multilayered media [1]. Monocytes infiltrate the intima and many differentiate into macrophages and lipid-loaded macrophage-like foam cells. In addition, there is the full or partial loss

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of the normal contractile phenotype of many SMCs to give a constellation of states called the synthetic SMC phenotype [2] in which SMCs become more rounded and mobile, begin to actively proliferate, lose their ability to contract, and increase synthesis of certain extracellular proteins. Phenotypic modulation of SMCs during development of atherosclerosis can be protective or disease-promoting [3,4].

Despite the importance of epigenetics to cell differentiation, there have been only a small number of reports about atherosclerosis-related epigenetic changes, and some of these studies focused on endothelial cells or non-aorta atherosclerosis [5]. A comparison of atherosclerotic human femoral artery samples with mammary artery samples as controls indicated disease-related DNA hypomethylation of gene and promoter regions [6]; however, non-disease related tissue-specific differences might be major contributors to the observed differential methylation. The most comprehensive studies of DNA methylation changes in human aorta examined pairs of atherosclerotic aorta and control aorta from the same individual using a microarray assay (15 sample pairs) or whole-genome bisulfite-sequencing (WGBS or bisulfite-seq; 1 pair of samples) [7,8]. Grade-independent differentially methylated sites (54625 in > 3000 genes) were predominantly disease-hypermethylated. However, in contrast to WGBS, microarray studies lack the power to resolve differentially methylated regions (DMRs) in most non-promoter regions, e.g., enhancers.

We re-evaluated the DNA epigenetics of atherosclerotic aorta using available WGBS data from previous studies [7,9] and much more stringent criteria for calculating DMRs to reflect mostly changes in SMCs. We also compared atherosclerosis-DMRs to normal tissue-specific DMRs, genome-wide chromatin modifications, and transcription profiles [9,10] to elucidate biological associations of the disease DMRs. In addition, we examined individual gene neighborhoods of interest because composite epigenetic analyses of genome-wide data can miss important insights into the likely functionality of DMRs [11]. We discovered a very strong preference for atherosclerosis-linked DNA hypermethylation at aorta-associated enhancer-like regions, including in genes well known to be associated with atherosclerosis.

2. Materials and methods

For the atherosclerotic and control aorta samples from the same individual (88 yo female, athero aorta A, aortic arch, and control aorta A, thoracic aorta), the bisulfite-seq data from Zaina et al. [7] were used. These data were supplemented with two additional control aorta bisulfite-seq profiles from a 34 yo male and a 30 yo female (control aortas B and C, respectively; Roadmap Epigenetics Project [9,12]). The atherosclerotic aorta sample included the muscular tissue underneath the plaque [7], which should consist predominantly of SMCs [13]. Determination of DMRs using the Uniform Product distribution [14], mapping them, and comparing them to normal epigenetic and transcriptomic databases are described in an expanded Methods section in a companion article in Data in Brief [15]. In Fig. 1, the gene subregions relative to the transcription start site (TSS) or end site (TES) are as follows: promoter, TSS -2 kb to TSS +0.2 kb; gene body, TSS +0.2 kb to TES +2 kb; intergenic, other positions.

3. Results

3.1. Atherosclerosis-associated hypermethylated DMRs were predominant over hypomethylated DMRs and often overlapped aorta enhancers

Previously, Zaina and coworkers [7] using one matched set of atherosclerotic aorta (grade VII, including underlying muscle) and uninvolved aorta from the same individual (athero aorta A and control aorta A) identified 54,625 DMRs with ≥5 consecutive and consistently differentially methylated CpG sites from WGBS data (Fisher exact test, $p < 0.05$) [7]. We re-determined atherosclerosis-associated DMRs using their downloaded data [16] but with much more stringent conditions and included WGBS data from two additional control aortas [9,16] (controls B and C). We required an absolute percent methylation difference (PMD) of > 20%, a length of > 250 bp and gaps < 200 bp as well as ≥5 differentially methylated sites per DMR and that the DMRs were significant in the paired samples (athero aorta A *vs.* control aorta A) as well as in the comparison of atherosclerotic aorta and three control aortas. With our criteria, there were only 9220 DMRs associated with 4535 genes (see Data in Brief Table 1 [15]). Zaina et al. also did a microarray analysis of 15 atherosclerotic/normal aorta pairs [7]. Of their 892 genes reported to have differentially methylated sites, 454 were associated with athero DMRs ascertained by our stringent criteria. Moreover, 15 out of 18 sites in nine genes (*HOXA2, HOXA9, HOXA11-AS, HOXC4, HOXC11, PDGFA, PLAT, PRRX1* and *PXDN*) that Zaina et al. verified as significantly athero hypermethylated or hypomethylated by pyrosequencing [7] overlapped DMRs in our study.

We found that 85% of all the athero DMRs were hypermethylated (athero hypermeth DMRs) and 15% hypomethylated (athero hypometh DMRs; Fig. 1A). About 44% of the genes with athero hypometh DMRs were also associated with athero hypermeth DMRs. This result might be explained, in part, by previous reports of tissue-specific DNA hypomethylation and hypermethylation being positively associated with DNA upregulation depending on the gene region, chromatin, and cell context [11]. We sorted the hypermethylated DMRs by PMD > 40% or PMD = 20–40% (1032 and 6819 DMRs, respectively) and the hypomethylated DMRs by PMD < -40% or PMD -20 to −40% (75 and 1294 DMRs, respectively; see Data in Brief Tables 1a–1d [15]). Leukocyte infiltration or clonal expansion of atypical SMCs into the plaque region during atherosclerosis [17,18] are unlikely to produce athero DMRs defined by a 20% absolute PMD threshold, and this is especially unlikely for DMRs with absolute PMDs of > 40%.

We also compared the two control aorta samples from the 30 and 34 yo individuals with the control aorta from the 88 yo atherosclerotic patient. Unlike the atherosclerotic vs. the control aorta DMRs from the 88 yo patient for which hypermethylation predominated, the 88 yo control vs. 30–34 yo controls had more hypometh DMRs (5868 DMRs) than hypermeth DMRs (4212 DMRs). These DMRs could be due to several factors, including biological differences and technical variation because the bisulfite sequencing was performed in different labs with different sequencing depths. However, this finding argues against age-related changes accounting for the predominance of hypermethylation over hypomethylation in the athero DMRs.

We examined chromatin state segmentation profiles, which use genome-wide histone modifications typically associated with promoter, enhancer, repressor, or actively transcribed chromatin [9]. Surprisingly, 53% of the athero hypermeth DMRs overlapped ≥50 bp of enhancer-type (enh) chromatin in normal aorta, and 64% of these DMRs at enh chromatin were in gene bodies (Figs. 1B, 2A and 3A and 4A). Only 12% of athero hypometh DMRs overlapped aorta enh chromatin (Fig. 1B).

3.2. Atherosclerosis-associated hypermethylated DMRs and hypomethylated DMRs are enriched in functionally different sets of genes

The functional associations [19] of genes linked to hypometh or hypermeth athero DMRs were very different (see Data in Brief Table 2a [15]). For example, actin-related gene ontology (GO) terms were highly over-represented among athero hypermeth DMRs but not among athero hypometh DMRs. Integrin signaling and focal adhesion were highly favored GO terms among genes linked to athero hypermeth DMRs (but not athero hypometh DMRs) and are related to the actin/integrin/extracellular matrix signaling pathway [20]. The strongest GO association of athero hypometh (but not hypermeth) DMRs was with genes encoding sequence-specific DNA binding proteins.

Athero hypermeth DMRs often overlapped not just enh chromatin, but also super-enhancers. Super-enhancers are especially long and strong enhancers and can be defined as regions of > 3 kb enriched in

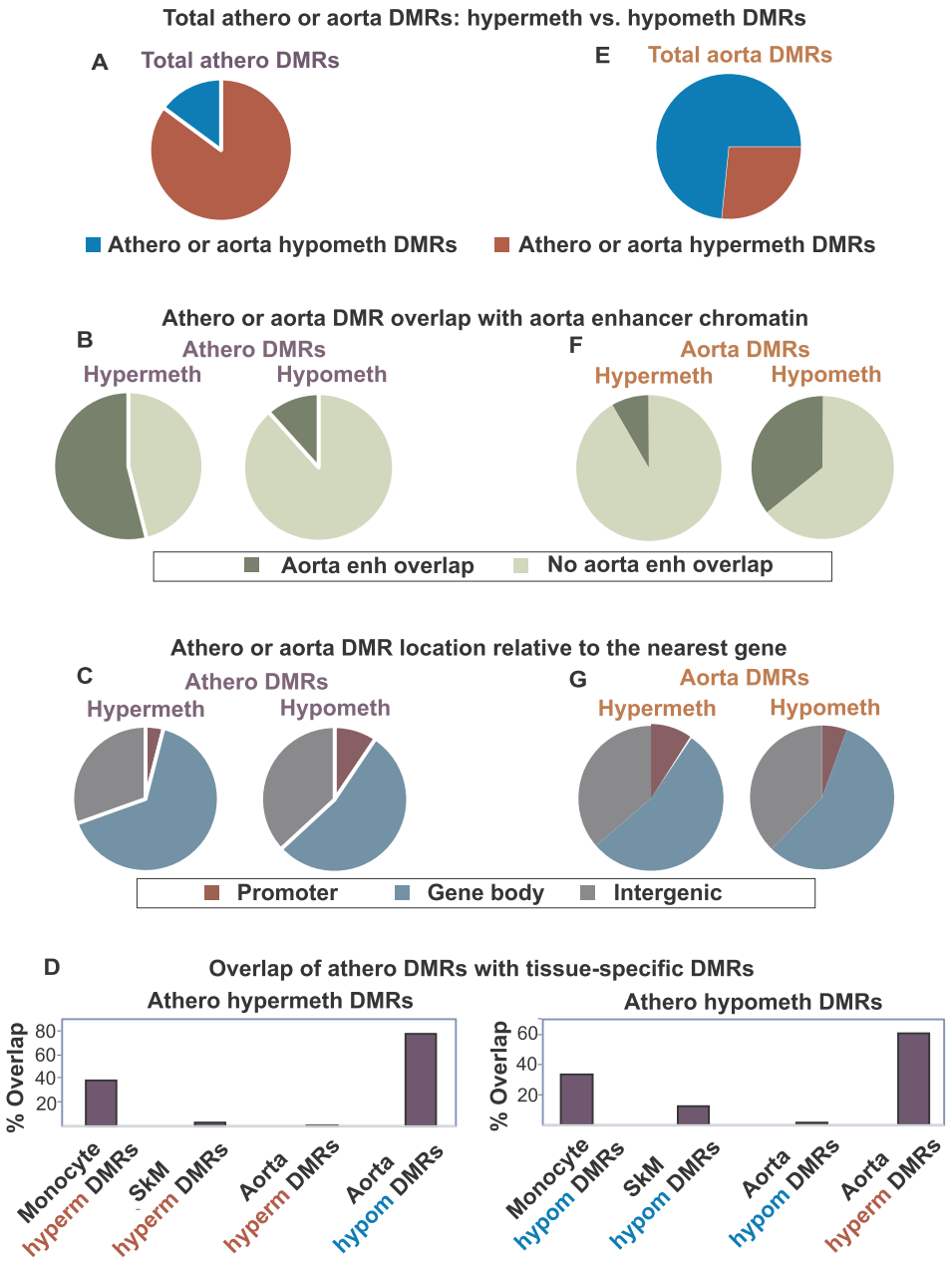

Fig. 1. Atherosclerosis-associated and normal aorta DMRs: genome-wide associations.
(A) Pie charts showing that athero DMRs were mostly hypermeth DMRs while aorta tissue-specific DMRs were mostly hypometh DMRs. (B) Athero hypermeth DMRs, but not athero hypometh DMRs, were greatly enriched in aorta enhancer chromatin. (C) Distribution of athero DMRs among gene regions. (D) Bar graphs showing the strong overlap of athero DMRs with aorta DMRs of the opposite directionality (athero hypermeth with aorta hypometh and *vice versa*) and athero DMRs overlap with monocyte DMRs of the same directionality. (E–G) parallel to (A–C) but for tissue-specific aorta DMRs (control aorta *vs.* heart/skeletal muscle/lung/adipose/monocytes), which indicated that the athero hypermeth DMRs are most similar to the aorta hypometh DMRs and *vice versa*. Enh, enhancer-type chromatin.

histone H3 lysine-27 acetylation (H3K27ac) [21–23]. A functional analysis [24] of genes associated with athero hypermeth DMRs and super-enhancers revealed, again, high enrichment in GO terms related to actin-binding (see Data in Brief Table 3 [15]).

3.3. Genome-wide analyses of atherosclerosis-associated DMRs indicate partial acquisition of a leukocyte-like epigenetic profile by atherosclerotic aorta

We identified aorta tissue-specific DMRs and monocyte-specific DMRs for comparison to athero DMRs using WGBS datasets [9] from normal aorta *vs.* those from monocytes, skeletal muscle (SkM), heart, lung and adipose tissues or from monocytes *vs.* those from aorta, SkM, heart, lung and adipose tissues. About 77% of athero hypermeth DMRs overlapped aorta hypometh DMRs, and 61% of athero hypometh DMRs overlapped aorta hypermeth DMRs (Fig. 1D). Aorta hypometh DMRs were much more plentiful than aorta hypermeth DMRs and much more likely to overlap enhancers (Fig. 1E and F). These findings suggest frequent atherosclerosis-linked increases in DNA methylation targeted to aorta hypometh DMRs at aorta enhancers.

Functional analyses also revealed associations of athero (disease-related) hypermeth DMRs and aorta (normal tissue-specific) hypometh DMRs. For example, the DNA sequence binding motif assigned to serum response factor (SRF), which plays a critical role in the development of vascular SMC [25], was strongly enriched at promoters for both athero hypermeth DMR genes and aorta hypometh DMR genes (see Data in

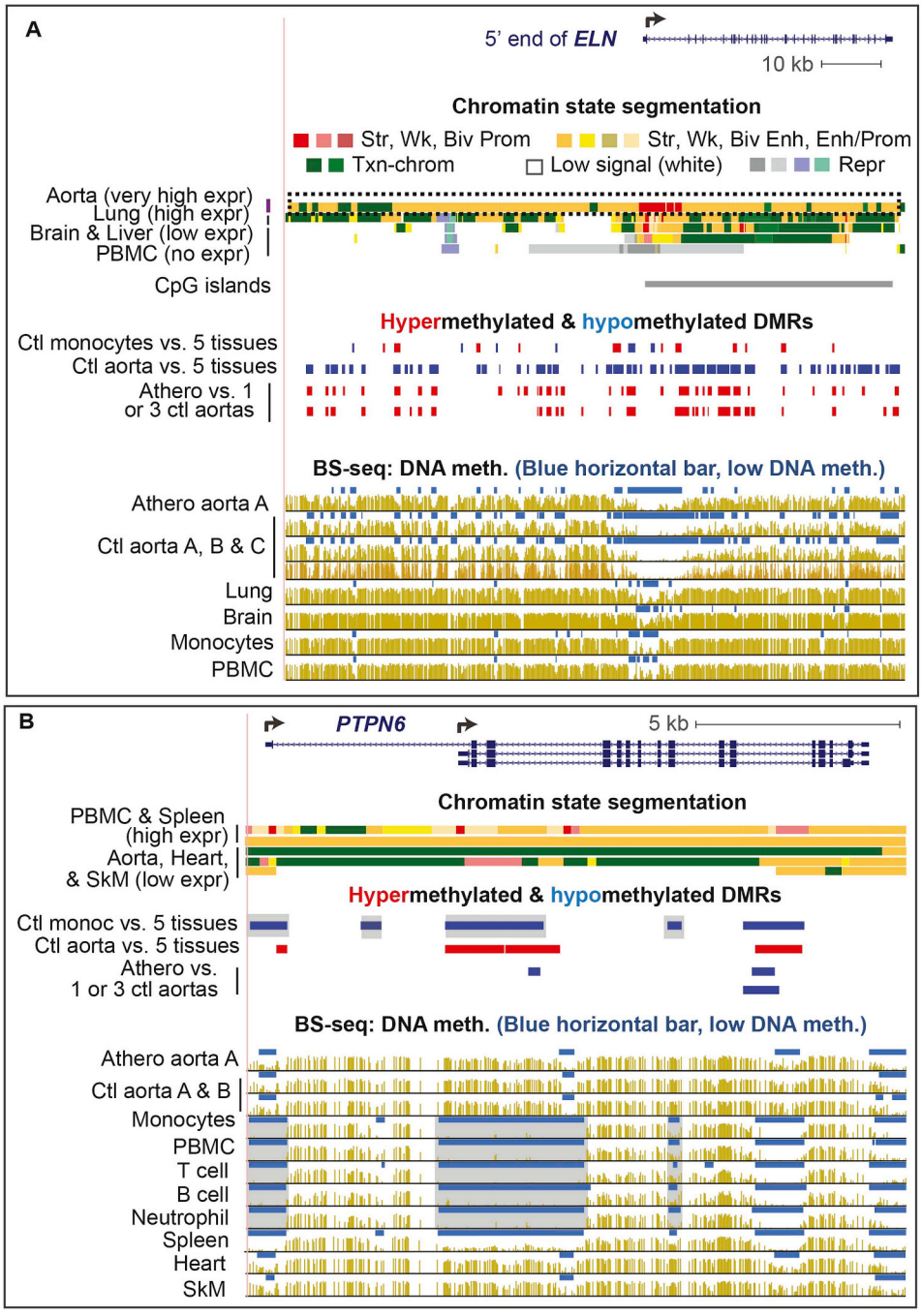

Fig. 2. Atherosclerosis-associated hypermethylation in the aorta-related *ELN* super-enhancer and hypomethylation in leukocyte-associated *PTPN6*.
(A) *ELN* displayed an aorta-specific super-enhancer (dotted box) that overlapped many athero hypermeth DMRs (red; bottom two DMR tracks) and aorta-specific (tissue-specific) hypometh DMRs (blue; middle DMR track; chr7:73,381,618–73,486,319). Bisulfite-seq is plotted as average % methylation at CpGs; blue horizontal bars, regions that display significant hypomethylation relative to the rest of the same genome [12]. (B) *PTPN6* displayed monocyte hypometh DMRs (chr12:7,055,252–7,071,384). Gray highlighting, monocyte DMRs with little or no athero hypomethylation. All tracks are aligned and derived from the UCSC Genome Browser with hg19 coordinates. Ctl, control; PBMC, peripheral mononuclear blood cells; brain, prefrontal cortex; heart, left ventricle; expr, expression; str, strong; wk, weak; biv, bivalent; prom, promoter; enh, enhancer; txn-chromatin, chromatin with histone marks of active transcription; repr, repressed.

Brief Table 2A [15]). Another example is that both athero hypometh DMRs and aorta hypermeth DMRs were very highly enriched at homeobox transcription factor (TF) genes.

In contrast, monocyte DMRs (but not SkM or heart DMRs) often overlapped athero DMRs with the same directionality, namely, monocyte hypermeth DMRs with athero hypermeth DMRs and monocyte hypometh DMRs with athero hypometh DMRs (Fig. 1D). While this finding might reflect the contribution of infiltrating monocytes to the observed athero DMRs, such a conclusion is refuted by the extent of DNA methylation differences as seen in strong athero DMRs (absolute value of PMD > 40%). Moreover, there was no significant enrichment specifically for leukocyte-related GO terms among strong athero DMRs (see Data in Brief Table 2B [15]). Lastly, epigenetic and transcriptomic profiles of many individual gene regions were inconsistent with athero DMRs being explained by monocyte (or any type of leukocyte) contamination. For example, the leukocyte-associated genes *PTPN6*, *CD79B,* and *SH3BP2* displayed monocyte hypometh DMRs that were also hypomethylated in other leukocyte cells types but mostly highly methylated in atherosclerotic aorta (Fig. 2B, gray highlighting, and see Data in Brief Figs. 1 and 2 [15]). All these findings argue against monocyte/macrophage infiltration explaining the observed athero DMRs.

3.4. Some of the hypermethylated DMRs are linked to key SMC genes previously implicated in atherosclerosis

Seven out of 14 synthetic phenotype markers and 12 of 18 contractile phenotype markers for SMCs [26] displayed athero DMRs (see Data in Brief Table 1 [15]). In addition, many of the 270 genes (see Data in Brief Table 4 [15]) that have been related to atherosclerosis

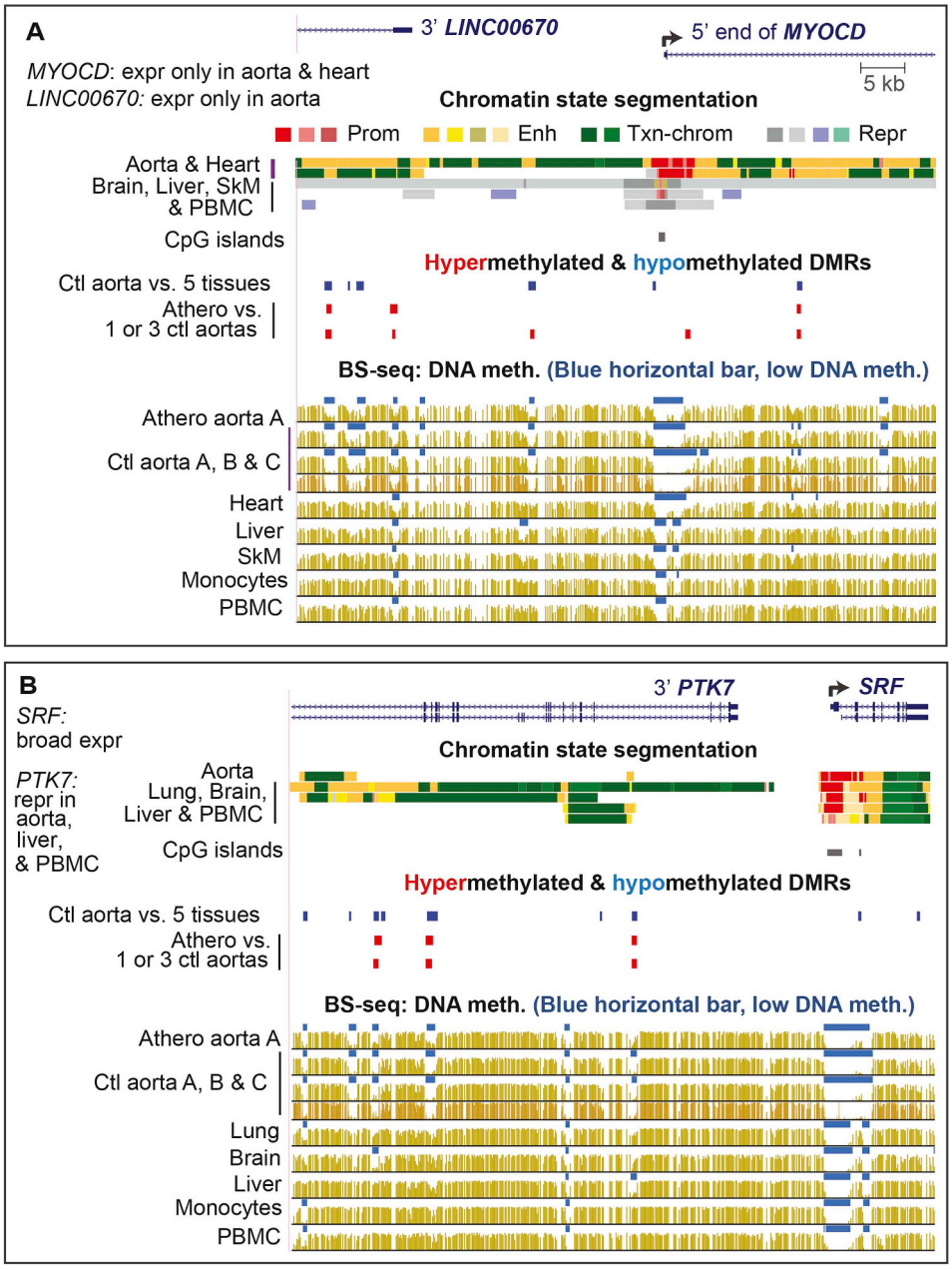

Fig. 3. *MYOCD* and *SRF* TF genes were associated with atherosclerosis-linked DNA hypermethylation but the hypermethylation linked to *SRF* was within the adjacent aorta-repressed *PTK7* gene.
(A) *MYOCD* was linked to athero hypermeth DMRs (chr17:12,527,074–12,600,390). (B) Aorta-specific hypomethylation overlapping athero hypermeth DMRs in *PTK7* gene (repressed in aorta) adjacent to *SRF* (broadly expressed; chr6:43,082,821–43,149,411). (Eight additional athero hypermeth DMRs in *MYOCD/LINC00670* gene region are shown in Data in Brief Table 1 [15]).

[27] were associated with athero DMRs. For example, *ELN,* which has 41 references linking it to the disease [27] and encodes an extracellular protein that stabilizes arteries, displayed multiple athero hypermeth DMRs. *ELN* is expressed at extremely high levels specifically in aorta, coronary artery and tibial artery (see Data in Brief Table 5 [15]). Its athero hypermeth DMRs were embedded in 103-kb super-enhancer, and most overlapped tissue-specific aorta hypometh DMRs (Fig. 2A, wide dotted box). Super-enhancers generally exhibit overall depletion in DNA methylation and are associated with very high gene expression [21,22,28]. *ACTA2*, *MYH11* and *MYH10*, which encode either actins or actin-binding myosins and were previously implicated in atherosclerosis (see Data in Brief Table 4 [15]), also exhibited multiple athero hypermeth DMRs that overlapped aorta super-enhancers or standard enhancers (see Data in Brief Figs. 3–5 [15]).

Among the transcription- or translation-regulatory genes linked to athero hypermeth DMRs and implicated in atherosclerosis are *MYOCD, SRF, SMAD3* and *C9orf3* (host gene for miR-23b and miR-27b; Figs. 3 and 4A). *SMAD3,* a broadly expressed gene, displayed athero hypermeth DMRs that suggest disease-related decreases in its aorta-specific pattern of enh chromatin or tissue-specific isoform usage related to epigenetic changes (see Data in Brief Fig. 6 [15]). *SMAD3* has been genetically linked to control of *COL4A1* and *COL4A2* [29], which also displayed multiple athero hypermeth DMRs.

MYOCD, a critical and specific TF for SMCs and heart, exhibited four athero hypermeth DMRs located within the gene (Fig. 3A and data not shown). Seven more were in or adjacent to the upstream non-coding RNA (ncRNA) gene *LINC00670* (see Data in Brief Table S1a and b [15]), which is expressed preferentially in arteries (see Data in Brief Table 5

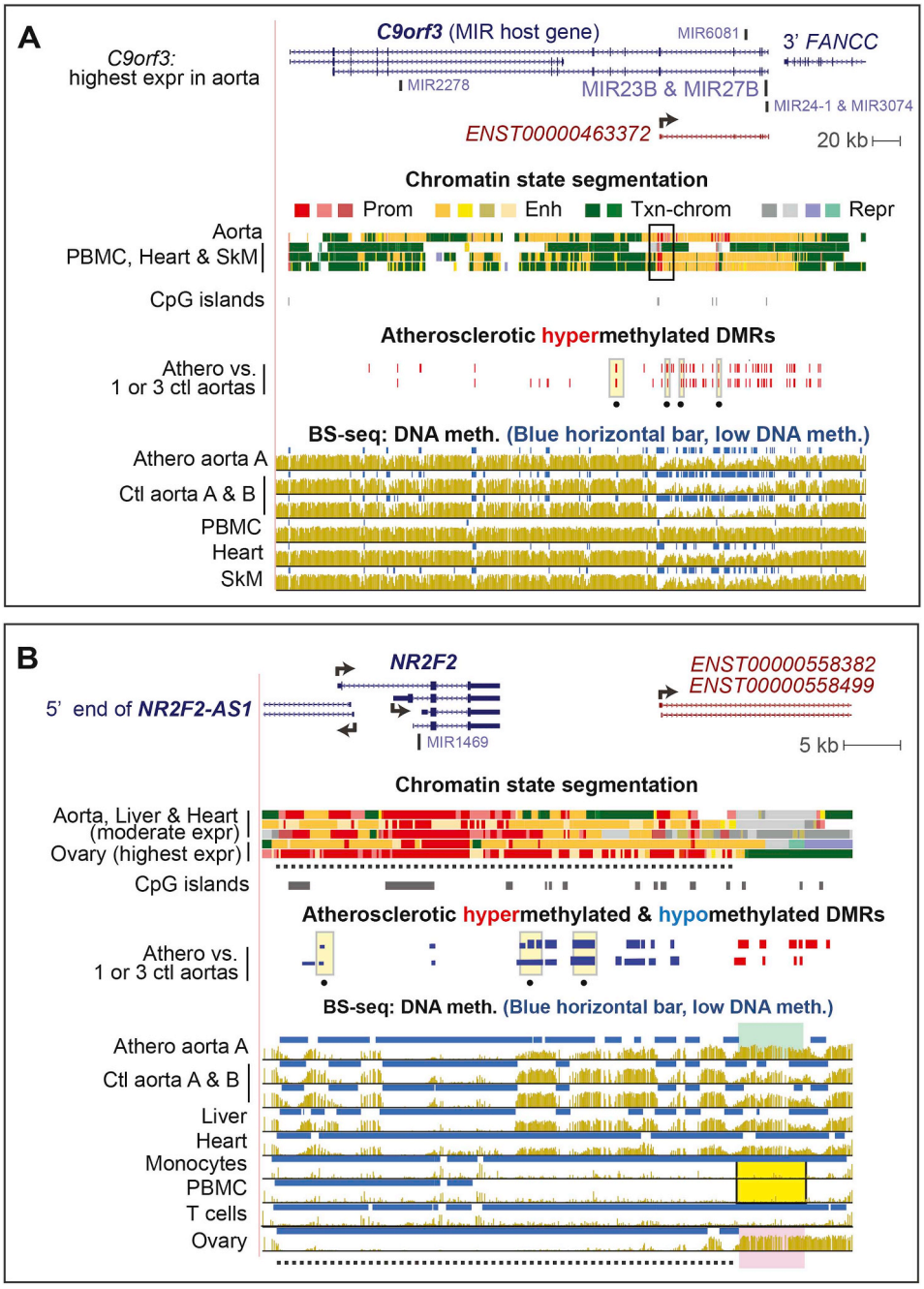

Fig. 4. Strong atherosclerosis-associated DMRs: *C9orf3*, the SMC-related miR-23b host gene, and *NR2F2*, a master developmental TF gene.
Some of athero DMRs in (A) *C9orf3* at chr9:97,478,939–97,923,541 and (B) near *NR2F2* (*COUP-TFII;* chr15:96,862,542–96,914,426) were strong DMRs (PMD > 40% or < -40%, respectively; yellow highlighting with a black dot underneath). PBMC have repressed chromatin throughout *NR2F2* neighborhood [11]. In (B): dotted line, hypomethylated ovary super-enhancer; green, yellow and pink highlighting, the region whose hypermethylation is positively associated with *NR2F2* expression.

[15]). MYOCD is a transcriptional co-activator of the broadly expressed TF SRF [30]. Although the *SRF* gene did not display DMRs, *PTK7*, which is upstream of *SRF,* had three athero hypermeth DMRs that overlap aorta hypometh DMRs (Fig. 3B). Because *PTK7* has very low expression in aorta, it is likely the aorta hypometh DMRs upregulate *SRF* rather than *PTK7* and so hypermethylation at these *PTK7* regions in atherosclerotic aorta could down-regulate *SRF*.

The athero hypermeth DMRs associated with *C9orf3* were especially plentiful (Fig. 4A; 23 DMRs, some of which had PMD > 40%, dotted yellow highlighting). This is the host gene for six miRNAs including miR-23b, which helps prevent SMC phenotypic switching in cultured cells [31]. Analysis of chromatin state profiles and isoform-specific GTEx data [10] suggests that an alternative promoter is used for transcription in aorta and many other tissues (black box, chromatin state segmentation; Fig. 4A). The cluster of athero hypermeth DMRs immediately downstream of this promoter could down-modulate its activity.

3.5. Unusual atherosclerosis-associated DMRs associated with genes encoding developmental transcription factors: TBX and HOX family genes and NR2F2

Many of the genes that have unusually dense clusters of DMRs or especially strong DMRs encode early developmental TFs, including homeobox *(HOX)* TF genes, T-box (*TBX*) TF genes, and *NR2F2* (*COUP-TFII*), a gene specifying a TF involved in many types of embryonic

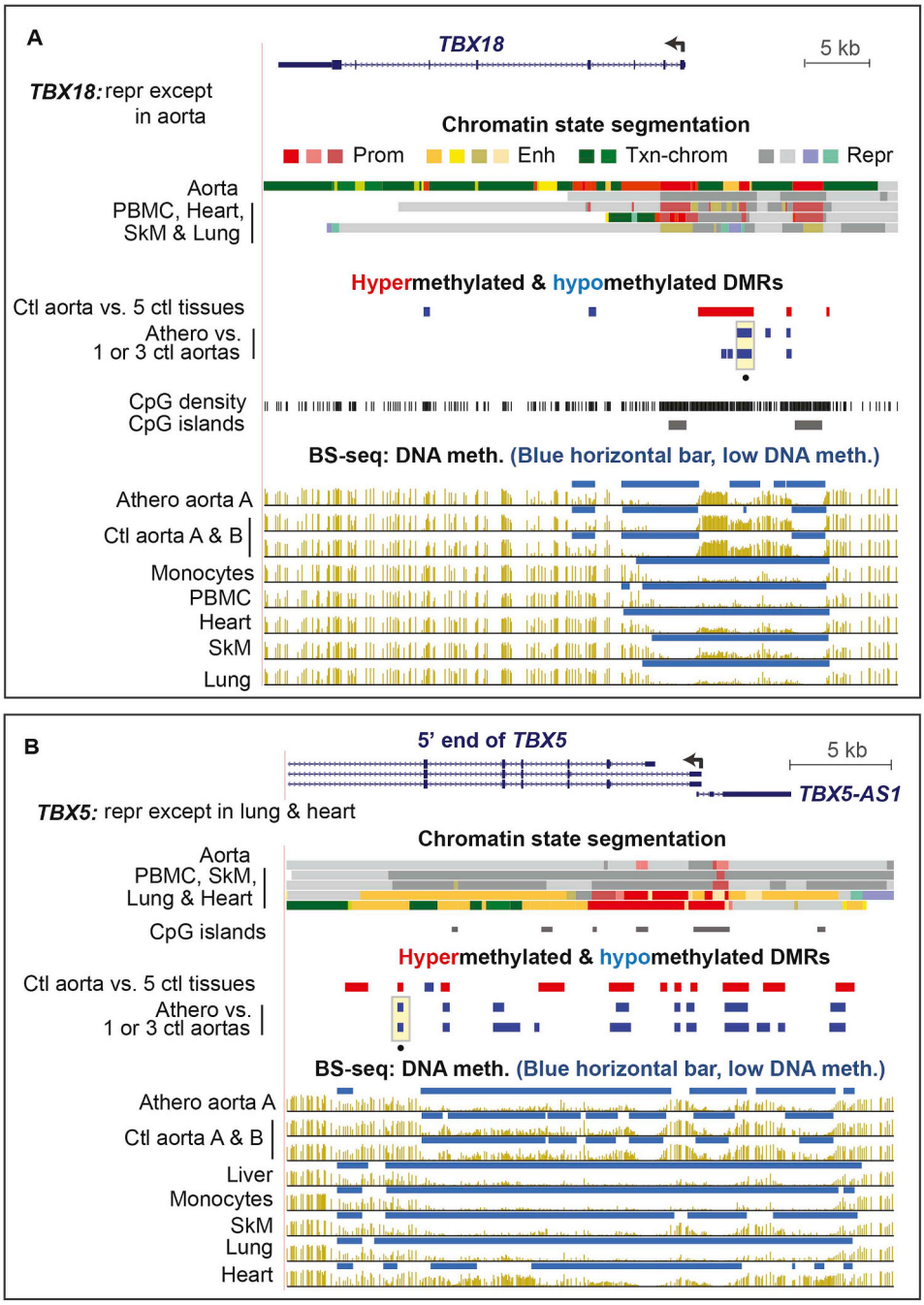

Fig. 5. Developmental TF genes, *TBX18* and *TBX5*, displayed atherosclerosis-associated hypomethylated DMRs even though *TBX5* is not expressed in aorta. (A) *TBX18* (chr6:85,441,091–85,490,593). (B) *TBX5* (chr12:114,825,763–114,855,728). Yellow highlighting as in Fig. 4. CpG density, CG dinucleotide positions to show regions with low CpG content that, therefore, cannot display CpG methylation.

organogenesis including vein formation [32] (see Data in Brief Table 1 [15]). Adjacent to *NR2F2* were clusters of hypometh DMRs and of hypermeth DMRs, and some of these DMRs overlapped two isoforms of a long downstream ncRNA gene (Fig. 4B). This ncRNA gene and an antisense ncRNA gene upstream of *NR2F2* have post-natal tissue-specific expression patterns very similar to those *NR2F2* itself (Fig. 4B; see Data in Brief Table 5 [15]). The athero hypermeth DMRs downstream of *NR2F2* displayed very low methylation in leukocytes (Fig. 4B, yellow highlighting), where *NR2F2* is repressed [11], and unusually high methylation in ovary (Fig. 4B, pink highlighting), where it is most highly expressed. This suggests that high methylation in this ncRNA gene region favors *NR2F2* expression. Atherosclerotic aorta also had DNA hypermethylation in this *NR2F2*-far downstream region (Fig. 4B, green highlighting), which, together with the athero hypometh DMRs closer to the gene, might indicate upregulation of *NR2F2* in atherosclerotic aorta.

Seven of the 13 *TBX* family genes exhibited athero hypometh DMRs (see Data in Brief Tables 3 and 4 [15]). *TBX5* (Fig. 5B) and *TBX20* (see Data in Brief Fig. 11 [15]) displayed athero hypometh DMRs in their 5' gene regions even though they are repressed in normal aorta. Some *HOX* genes that are silent in aorta displayed athero DMRs (see Data In Brief Figs. 7–10 [15]). In contrast, *TBX18* had an athero hypometh DMR in the promoter-upstream region (Fig. 5A) and is expressed moderately in normal aorta. Therefore, while some of the athero DMRs associated with *TBX* or *HOX* family genes might affect transcription in cis, others are unlikely to do so because those genes are tightly repressed in the

majority of adult tissues, including control aorta.

4. Discussion

In this report, we present evidence that many of the athero hypermeth DMRs and the much less frequent hypometh DMRs might facilitate gene expression changes that could drive SMC phenotypic modulation and pathogenesis and that the athero hypermeth DMRs often occur at aorta enhancers. Our threshold of a 20% methylation difference for scoring a disease-associated DMR resulted in DMRs that should mostly reflect epigenetic alterations in SMCs, the dominant cell type in aorta. Nevertheless, we found a large overlap of the disease-linked DMRs with tissue-specific monocyte DMRs. This is probably due to phenotypic conversion and transdifferentiation of SMCs. Such leukocyte-like conversions can occur even while the cells still express markers of the contractile phenotype, although usually at a subnormal levels [3,17,33]. Similarly, we found that for athero DMRs that overlapped monocyte DMRs, the associated genes usually acquired only some of the monocyte hypo- or hypermethylation. This was even the case for genes that were preferentially expressed in monocytes and other leukocytes.

Athero hypermeth DMRs overlapped tissue-specific aorta hypometh DMRs even more frequently than they overlapped monocyte hypermeth DMRs. The over-representation of athero hypermeth DMRs at aorta hypometh DMRs could be explained by these athero DMRs frequently forming in regions of aorta enhancer-type chromatin. This suggests that de novo DNA methylation during atherogenesis is targeted preferentially to cis-acting regulatory regions, especially aorta enhancers. Given the magnitude of methylation changes that we found, the de novo DNA methylation probably occurred mostly in SMCs in the medial layer of atherosclerotic aorta. Chapelle et al. [18] demonstrated that clonal expansion of SMCs from the media is associated with their migration to the neointima and to the plaque. However, many of the SMCs left behind in the media, especially those close to the plaque, lost the elongated morphology characteristic of the contractile phenotype and displayed strong decreases in expression of the contractile marker *ACTA2*, which was among the genes that we found to have athero hypermeth DMRs. Epigenetic change in a considerable fraction of the medial SMCs would explain how we observed > 40% DNA methylation increases or decreases in many DNA regions in atherosclerotic aorta.

A caveat in this analysis is that the matched atherosclerotic and non-atherosclerotic aorta samples came from different parts of the aorta, aortic arch and thoracic aorta, respectively [7], which have different embryological origins [33]. In addition, the endothelial layers of these two different sections of aorta are subject to different flow dynamics that can alter their epigenetics [34,35]. The two additional control aortas used for DMR determination were from abdominal aorta or an unspecified portion of aorta. Differences in the embryological origin of curved (atherosclerotic sample) and linear aorta segments (at least two of the three control samples) could result in aorta region-specific DMRs [36–38] that arise during prenatal development but persist in adulthood whether or not they have a postnatal function. This is especially relevant to consideration of the numerous DMRs in *HOX* genes because of their role in shaping the body-plan early in embryogenesis. However, *HOXA6* and *HOXA9* were previously shown to be upregulated in atherosclerotic vs. control aorta and to display atherosclerosis-associated hypomethylation at tested CpG sites [7]. Their athero hypometh DMRs might favor transdifferentiation [39] to an osteochondrocytic phenotype (see Data in Brief Fig. 7 and Table S2c [15]).

Among genes associated with athero hypermeth DMRs, there was a strong over-representation of atherosclerosis-relevant GO terms, including actin cytoskeleton organization, smooth muscle contraction, focal adhesion, cell death, and the TGFβ-receptor signaling pathway, which is related to SMC phenotypic switching [40]. *ELN,* which was associated with many athero hypermeth DMRs, encodes a major structural protein in aorta relevant to atherosclerosis (see Data in Brief Table 4 [15]). The importance of fine-tuned control of *ELN* levels in aorta is evidenced by the finding that inactivating mutations in a single allele of the gene are linked to supravalvular aortic stenosis syndrome, which includes a congenital narrowing of part of the aorta [41]. We hypothesize that athero hypermeth DMRs in the aorta-specific super-enhancer of *ELN* down-modulate its enhancer activity and, thereby, decrease the rate of transcription of this gene, given the known link [11,28,42] between DNA hypomethylation and enhancers (Fig. 1F).

Other examples of athero hypermeth genes with known relationships to atherosclerosis are *COL4A1* and *COL4A2,* whose protein products are present in subnormal levels in the medial layer of atherosclerotic tissue [29]. Transcription of the genes encoding these proteins is positively regulated by SMAD3, a signaling TF [43], which also is encoded by a gene that exhibited athero hypermeth DMRs (see Data in Brief Fig. 6 [15]). Two other TF-encoding genes whose athero hypermeth DMRs could play a major role in atherosclerosis are *MYOCD* and *SRF*, which code for subunits of a MYOCD/SRF TF complex that induces expression of most SMC contractile phenotype marker genes [30], including athero hypermeth *ACTA2* and *MYH11*. These findings illustrate how multifaceted control of gene expression might drive phenotypic change in atherosclerotic aorta SMCs.

Our study also provides epigenetic clues for roles in atherosclerosis of genes that have been little studied in regard to this disease, e.g., *NR2F2*, the master organogenesis TF and the developmental TF gene, *TBX18*. *TBX18* is implicated in the generation of SMCs during development [40] and was reported to be induced in the endothelial layer of atherosclerotic arteries [44]. *NR2F2* is associated with phenotypic switching in vein endothelial cells [45]. These two genes displayed athero hypometh DMRs which suggests disease-linked upregulation of expression because they overlap a promoter or enhancer-type chromatin region in aorta.

A possible explanation as to why atherosclerosis-associated DNA hypermethylation greatly exceeds hypomethylation for DMRs comes from recent studies of TET2, a 5-methylcytosine (5 mC) dioxygenase. Decreased levels of TET2 in SMC, endothelial cells, and leukocytes have been implicated in atherosclerosis [46–48]. One of the functions of TET2 is participation in a DNA demethylation pathway (5 mC → C) through a 5-hydroxymethylcytosine intermediate [49]. Down-regulation of *TET2* can limit DNA demethylation resulting in increased genomic 5 mC levels by perturbing a dynamic DNA methylation/demethylation cycle that is especially targeted to regulation of enhancer activity [42,50]. TET2 down-regulation by itself or possibly in conjunction with increased DNA methyltransferase activity might drive predominant DNA methylation gain in atherosclerosis. Importantly, Liu et al. [46] showed that experimentally introduced decreases in TET2 could down-regulate *MYOCD, MYH11,* and *SRF* but ascribed this result just to effects of increased promoter methylation. Our finding of frequent atherosclerosis-associated DNA hypermethylation at aorta enhancer-type chromatin, including at these three genes and at the miR-145 host gene [51], suggests that enhancer hypermethylation also probably contributes to down-regulating contractile phenotype SMC genes during pathogenesis.

Conflicts of interest

The authors declare that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Study design was conceived by ME and ML. Biostatistical analyses and mapping of DMRs were by ML and CB. Analysis of individual gene regions was by KCE and ME. Manuscript preparation was mostly by ME with editing by KCE.

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