Embryological-Origin–Dependent Differences in Homeobox Expression in Adult Aorta

Role in Regional Phenotypic Variability and Regulation of NF-κB Activity

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Objective—Different vascular beds show differing susceptibility to the development of atherosclerosis, but the molecular mechanisms underlying these differences are incompletely understood. This study aims to identify factors that contribute to the phenotypic heterogeneity of distinct regions of the adult vasculature.

Approach and Results—High-throughput mRNA profiling in adult mice reveals higher expression of the homeobox paralogous genes 6 to 10 (*Hox6-10*) in the athero-resistant thoracic aorta (TA) than in the athero-susceptible aortic arch (AA). Higher homeobox gene expression also occurs in rat and porcine TA, and is maintained in primary smooth muscle cells isolated from TA (TA–SMCs) compared with cells from AA (AA–SMCs). This region-specific homeobox gene expression pattern is also observed in human embryonic stem cells differentiated into neuroectoderm–SMCs and paraxial mesoderm–SMCs, which give rise to AA–SMCs and TA–SMCs, respectively. We also find that, compared with AA and AA–SMCs, TA and TA–SMCs have lower activity of the proinflammatory and proatherogenic nuclear factor-κB (NF-κB) and lower expression of NF-κB target genes, at least in part attributable to HOXA9-dependent inhibition. Conversely, NF-κB inhibits *HOXA9* promoter activity and mRNA expression in SMCs.

Conclusion—Our findings support a model of Hox6-10–specified positional identity in the adult vasculature that is established by embryonic cues independently of environmental factors and is conserved in different mammalian species. Differential homeobox gene expression contributes to maintaining phenotypic differences between SMCs from athero-resistant and athero-susceptible regions, at least in part through feedback regulatory mechanisms involving inflammatory mediators, for example, reciprocal inhibition between *HOXA9* and NF-κB. **(*Arterioscler Thromb Vasc Biol*. 2013;33:1248-1256.)**

Key Words: atherosclerosis ■ homeobox ■ NF-κB ■ phenotypic diversity ■ smooth muscle cell

Animal and human studies have conclusively established that different blood vessels and distinct segments within a given artery are heterogeneous in their susceptibility to developing vascular pathologies in response to common risk factors.[1–3] Differences in flow patterns and hemodynamic forces within the vascular system are thought to play a major role in establishing regional heterogeneity in the susceptibility to vascular disease initiation and progression.[4–6] For example, vascular regions with low-shear stress or oscillatory and turbulent shear stress, such as regions of curvature and branching, are more susceptible to atherosclerotic plaque formation. In contrast, straight vessel segments with laminar blood flow are relatively resistant to atherosclerosis development. Moreover, there is evidence that, independently of hemodynamic factors, intrinsic cellular features that in part reflect distinct embryonic origins also contribute to establishing heterogeneous vascular responses to particular biological or pathological stimuli in adult organisms.[7,8] For example, using a canine model of homograft transplantation, Haimovici and Maier[9,10] found that atherosclerosis-prone abdominal aortic segments transplanted into atherosclerosis-resistant regions develop atherosclerosis, and that conversely, atherosclerosis-resistant vessels fail to develop intimal lesions when transplanted into atherosclerosis-prone positions in the abdominal aorta. It is also noteworthy that some regional heterogeneity of the vascular system is maintained in vitro.[7,8] For example, cell proliferation and migration, both of which contribute to atherosclerosis development,[11,12] are greater in primary smooth muscle cells (SMCs) derived from atherosclerosis-prone coronary artery and aortic arch (AA) than in SMCs from athero-resistant femoral arteries.[13,14]

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Previous studies demonstrated variation in susceptibility to atherosclerosis among inbred strains of wild-type mice fed an atherogenic diet, with C57BL/6J being the most susceptible.[15] Disease progression is aggravated in apolipoprotein E knockout mice (apoE-KO), which show impaired clearing of plasma lipoproteins and develop atherosclerosis in a short time.[16] In the present study, we performed high-throughput transcriptomic studies in aorta of young C57BL/6J wild-type and apoE-KO mice fed low-fat standard chow to identify novel mediators that contribute to establishing phenotypic differences between the highly atherogenic AA and the atheroresistant thoracic aorta (TA) at prelesional stages. We focused on genes displaying differential expression in AA compared with TA in both wild-type and apoE-KO mice, because these genes are more likely to be regulated by positional cues independently of genotype. We demonstrate that transcription factors of the homeobox gene family show significant differences in mRNA and protein expression between adult TA and AA in wild-type and apoE-KO mice. Homeobox genes encode homeodomain-containing transcription factors that are master regulators of embryonic development and specify cell fates along the anterior-posterior axis of all bilaterian embryos from a wide range of evolutionarily very distant animal species.[17–20] The class I vertebrate homeobox genes (*HOX* in humans and pigs, *Hox* in mice and rats) include 39 members organized in 4 paralogous clusters (*Hoxa, Hoxb, Hoxc, and Hoxd*) that are located in different chromosomes (Figure 2B). During development, *Hox* genes are expressed in a temporally and spatially colinear pattern, with the most 3′ members of each cluster being expressed earlier in embryonic development and predominantly in anterior regions, whereas the most 5′ genes are activated later, mainly in posterior tissues.[17–20] We report that differences in homeobox gene expression between TA and AA found in the mouse are conserved in rats and pigs, and are maintained in primary adult SMCs and in developmental-origin–specific human SMCs cultured under static conditions. We also provide evidence that differences in *Hoxa9* expression in distinct vessel segments contribute to establishing differential activity of the proatherogenic nuclear factor-κB (NF-κB), which in turn represses *Hoxa9* expression.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Global Analysis of Gene Expression in Mouse Aortic Arch and Thoracic Aorta

To identify factors involved in establishing vascular heterogeneity that may contribute to atherosclerosis development in adulthood, we performed high-throughput transcriptomic analysis of athero-susceptible AA and athero-resistant TA isolated from young wild-type and apoE-KO mice fed control chow. As expected, wild-type mice were normocholesterolemic, and apoE-KO mice exhibited mild hypercholesterolemia (Figure 1A). Consistent with a very early stage of atherosclerosis development, atherosclerotic lesions in the aortic sinus of apoE-KO mice were absent or very incipient, as demonstrated by immunohistochemical analysis with macrophage-specific anti–Mac-3 antibody (Figure 1B), and atherosclerotic lesions were completely absent from wild-type mice (data not shown). We hybridized labeled aRNA from pooled AA and TA to

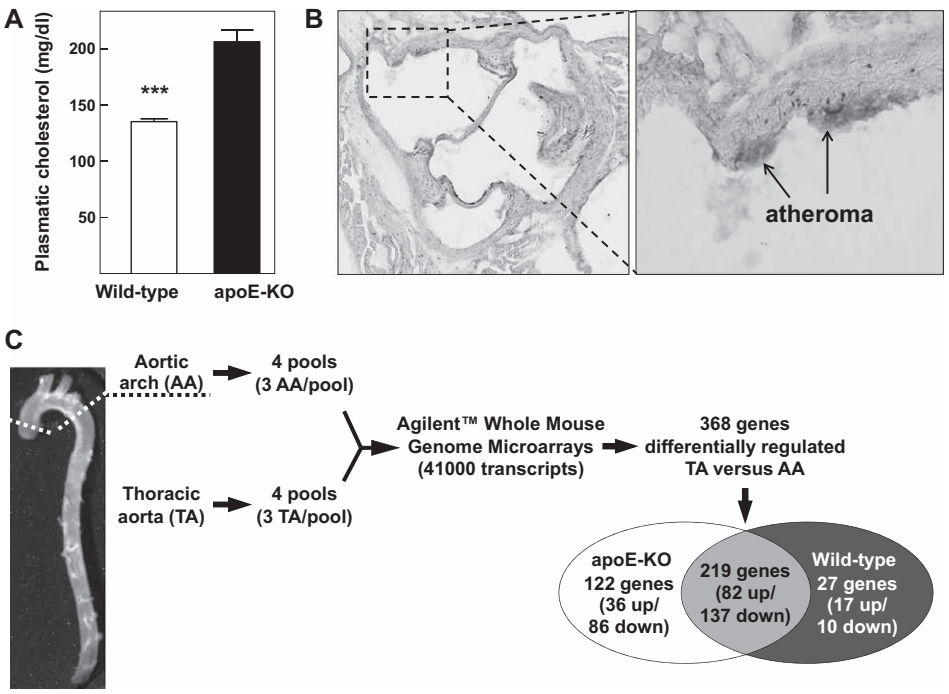

Figure 1. Rationale and design of high-throughput analysis of atherosclerosis-related gene expression in mouse aorta. Three-month-old wild-type and apolipoprotein E knockout (apoE-KO) mice were fed low-fat standard chow. **A**, Total plasma cholesterol (n=6 mice per genotype). **B**, Representative images of macrophage-specific anti–Mac3 immunostaining showing incipient atherosclerotic lesions in aortic sinus of apoE-KO mice. **C**, Aortic arch (AA) and thoracic aorta (TA) were extracted from 12 mice of each genotype, and tissue was pooled to isolate total RNA for microarray studies. Using the criteria described in Material and Methods, 122 transcripts exhibited differential regulation between TA and AA only in apoE-KO mice (Table II in the online-only Data Supplement), 27 transcripts only in wild-type mice (Table III in the online-only Data Supplement), and 219 transcripts in mice of both genotypes (Table IV in the online-only Data Supplement).

Agilent Whole Mouse Genome Microarrays (Figure 1C). After normalization and filtering of data as described in Materials and Methods, we focused on differentially expressed genes (adjusted to *P* value ≤0.05) showing differences in expression of at least 2-fold between TA and AA for at least 1 genotype. Using these criteria, we identified a total of 368 genes differentially expressed in TA compared with AA (Figure 1C), of which 122 genes were differentially expressed only in apoE-KO mice (36 upregulated and 86 downregulated; Table II in the online-only Data Supplement), 27 genes were differentially expressed only in wild-type mice (17 upregulated and 10 downregulated; Table III in the online-only Data Supplement), and 219 genes were differentially expressed in both genotypes (82 upregulated and 137 downregulated; Table IV in the online-only Data Supplement; Figure 1C). Table V in the online-only Data Supplement shows the functional classification of all the genes differentially expressed between TA and AA according to the Biological Process gene ontology terms (level 1) using the online software The Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7).

Differential Regulation of Class I Homeobox Genes in Mouse Aorta

We focused on genes that were differentially expressed between TA and AA in both wild-type and apoE-KO mice, because these are more likely to be regulated by positional cues independently of genotype. We noted that a subset of 11 TA-enriched genes in the Developmental Processes category were homeobox genes (Table V in the online-only Data Supplement). Indeed, the most upregulated gene in our microarray analysis is *Hoxc9*, and 6 of the 10 most upregulated transcripts in TA also correspond to *Hox* genes (Table IV in the online-only Data Supplement). The magnitude of the increase in *Hox* expression is similar for both genotypes (Figure 2A), consistent with the notion that they are truly regulated in a region-dependent manner and independently of the genotype. Our microarray studies also reveal that *Hoxb8* and *Hoxc8* are expressed at higher level in TA than in AA in apoE-KO mice, but not in wild-type mice (Figure 2A).

Consistent with the spatial colinearity that characterizes the *Hox* cluster genes during embryonic development,[17–20] all the *Hox* genes that are expressed at higher level in mouse TA than in AA, with the sole exception of *Hoxd4*, belong to paralogous groups 6 to 10 of the 4 *Hox* clusters (Figure 2B). Quantitative polymerase chain reaction (qPCR) analysis of fresh RNA preparations (4 pools each containing 3 AAs or 3 TAs) confirmed higher expression of *Hox6-10* transcripts in TA from wild-type mice (Figure 3A, left), including some members of these paralogous groups which were either not detected in wild-type tissue or did not pass the selection criteria in the microarray analysis. Western blot analysis of 3 independent pools of aortic tissue confirmed higher expression of Hoxa9 and Hoxc8 proteins in mouse TA than in AA (Figure 3B). In contrast, expression of the 3′-most genes *Hoxa1* and *Hoxa2* did not differ between AA and TA (Figure I in the online-only Data Supplement). These results show that spatial colinearity is also a characteristic of *Hox* gene expression in the adult mouse aorta.

To ascertain whether differential *Hox* expression is maintained in culture, we performed quantitative polymerase chain reaction in primary SMCs isolated from mouse AA (AA–SMCs) and TA (TA–SMCs). These studies revealed higher *Hox6-10* expression in TA–SMCs than in AA–SMCs, although *Hoxd8* differential expression was not statistically significant (Figure 3A, right).

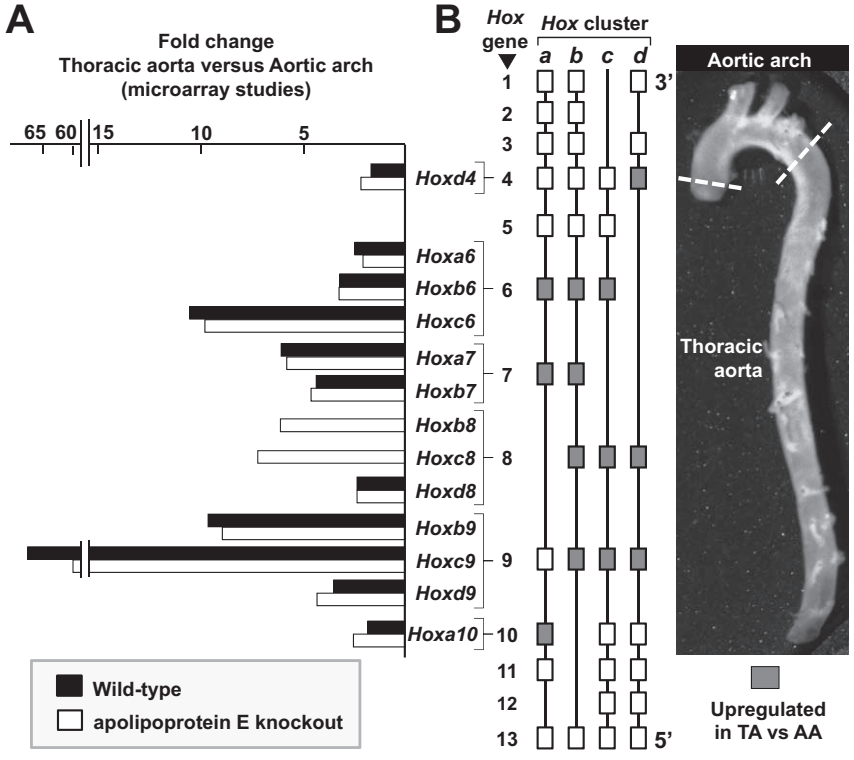

Figure 2. Homeobox gene expression in mouse aorta. **A**, Microarray studies revealed higher level of expression of 13 homeobox genes in thoracic aorta than in aortic arch (11 in both genotypes, and *Hoxc8* and *Hoxb8* only in apolipoprotein E knockout mice). **B**, Representation of the 4 homeobox clusters (a-d) and individual *Hox* genes (1–13), showing members that are expressed at similar levels in AA and TA (white boxes) or upregulated in TA vs AA (grey boxes).

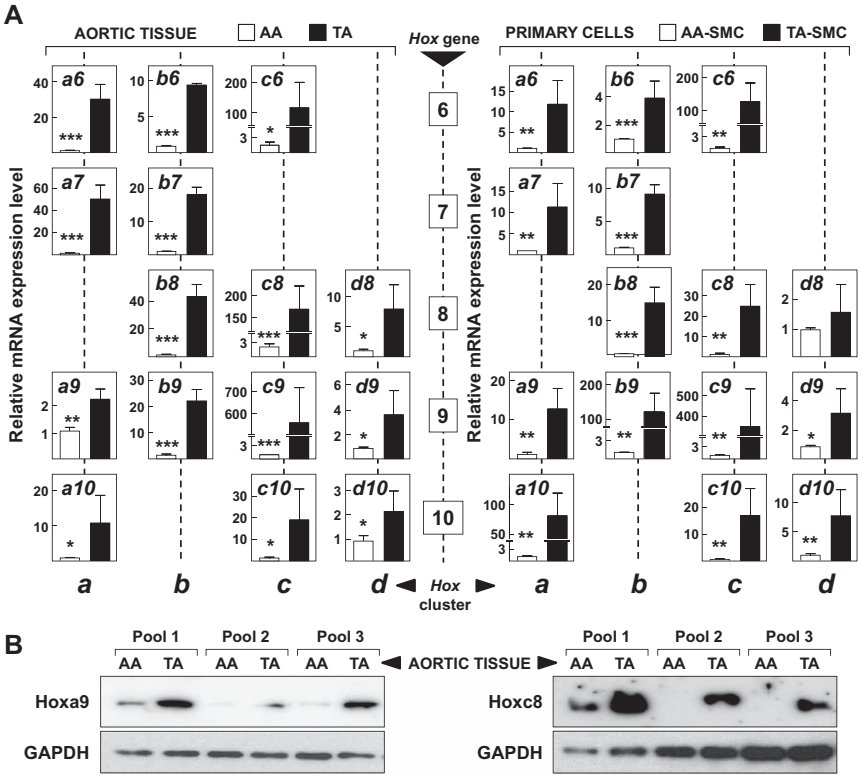

Figure 3. Homeobox expression in mouse aortic tissue and primary smooth muscle cells (SMCs). Three-month-old wild-type mice fed standard chow were euthanized, and total RNA or protein was isolated from aortic tissue (pools of 3 aortic arches [AAs] or thoracic aortas [TAs]) or primary SMCs (established in culture from pools of 5 AAs or TAs). **A**, *Hox* gene expression quantified by quantitative polymerase chain reaction (qPCR) using 18S as an internal control. Results are presented in arbitrary units normalized to expression in AA (=1). Data are mean±SEM of 4 independent extractions for mouse aortic tissue and 5 independent extractions for mouse SMCs, measured in triplicate. **P*<0.05; ***P*<0.01; ****P*<0.001. **B**, Hoxa9 and Hoxc8 protein expression in lysates from 3 independent pools analyzed by Western blot. GAPDH was used as loading control.

Regional Differences in Homeobox Gene Expression in Aorta Are Conserved in Rats and Pigs

To determine whether the *Hox* expression pattern in the adult vasculature is conserved in mammals, we performed mRNA expression studies in aorta from rats and pigs. As in the mouse models, *Hoxc8*, *Hoxa9*, and *Hoxc9* are expressed at higher level in rat TA and TA–SMCs than in AA and AA–SMCs (Figure II in the online-only Data Supplement). In contrast, expression of the 3′-most genes *Hoxa1* and *Hoxa2* is lower in rat TA and TA–SMCs (Figure I in the online-only Data Supplement). Higher levels of *HOXC8*, *HOXA9*, and *HOXC10* expression in TA than in AA were also detected in pig (Figure II in the online-only Data Supplement).

Human Neuroectoderm–Derived and Paraxial Mesoderm–Derived Smooth Muscle Cells Exhibit Differential Homeobox Gene Expression

The vasculature is formed by SMCs from different embryological origins.[7,8] Lineage mapping studies have shown that neural crest cells give rise to AA–SMCs, whereas TA–SMCs derive from paraxial mesoderm.[7,21] Therefore, we examined *HOX* expression in human neuroectoderm–derived SMCs (NE–SMCs) and paraxial mesoderm–derived SMCs (PM–SMCs) differentiated from pluripotent human embryonic stem cells (Figure 4A).[22] These in vitro–derived cells express markers of differentiated SMCs and recapitulate the unique proliferative and secretory responses to cytokines characteristic of their in vivo aortic SMC counterparts of distinct embryonic origin.[22] In agreement with our analysis in animal models, microarray studies showed higher expression of *HOXB6, C6, B7, B8, C8, A9, C9, A10*, and *D10* genes in human PM–SMCs than in NE–SMCs (Figure 4B). Higher expression of *HOXC6*, *B7*, *C8*, *A9*, and *A10* in PM–SMCs than in NE–SMCs was confirmed by quantitative polymerase chain reaction (Figure 4C). In contrast, *HOXA1* was expressed at similar levels in both cell types, and *HOXA2* was downregulated in PM–SMCs (Figure I in the online-only Data Supplement). In vitro–derived NE–SMCs and PM–SMCs thus recapitulate the differences in *HOX* expression pattern observed in adult AA and TA.

Negative Feedback Regulation Between HOXA9 and NF-κB in Smooth Muscle Cells

Atherosclerosis is a chronic inflammatory disease,[23] and the transcription factor NF-κB plays an important role in the process of vascular inflammation.[24] Previous studies demonstrated that NF-κB is activated in the endothelium of athero-susceptible regions,[25,26] and is downregulated by HOXA9 in human endothelial cells.[27] Consistent with these findings, our microarray studies show lower expression of the NF-κB targets *E-selectin*, *galectin 3*, and *cxcl5* in the atheroresistant TA. However, it remains to be established whether HOXA9 and NF-κB are functionally related in SMCs, and whether such a relationship contributes to differential expression or activity of these transcription factors in the adult vasculature. To tackle this question, we performed electrophoretic mobility shift assays on extracts of aortic tissue or primary SMCs. Incubation of mouse aortic extracts with a radiolabeled probe containing the consensus DNA–binding site for NF-κB generated a retarded band that was efficiently competed out with an excess of unlabeled consensus oligonucleotide, but not with an equivalent molar excess of the mutated sequence (Figure 5A, lane 1–3). This retarded

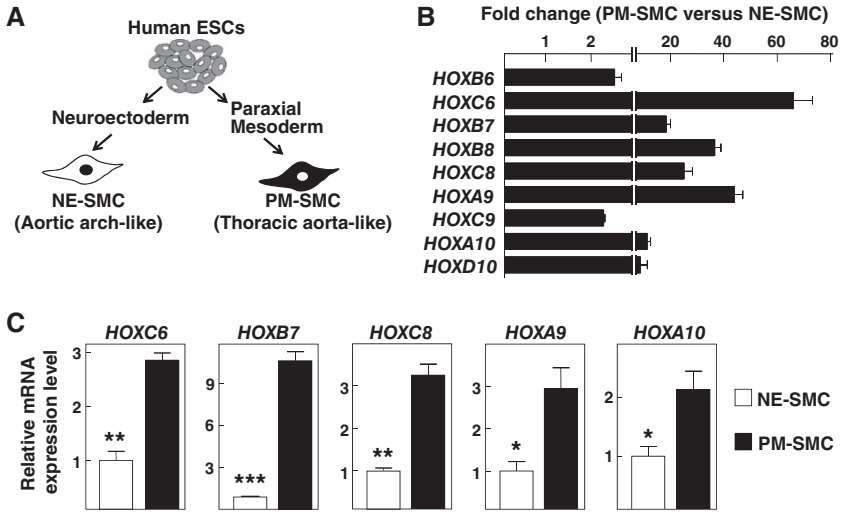

Figure 4. Homeobox gene expression in in vitro–derived human neuroectoderm- and paraxial-mesoderm–derived smooth muscle cells (SMCs). **A**, Scheme of in vitro derivation of neuroectoderm-derived SMCs (NE–SMCs) and PM–SMCs from human embryonic stem cells (hESCs). **B**, *HOX* genes upregulated in paraxial mesoderm–derived (PM–SMCs) compared with NE–SMCs, as revealed by microarray analysis. **C**, Quantitative polymerase chain reaction (qPCR) analysis of *HOX* gene expression. Results are presented in arbitrary units normalized to NE–SMCs (mean±SEM; n=3). **P*<0.05; ***P*<0.01; ****P*<0.001.

band was supershifted on incubation with an antibody against the NF-κB subunit p65, but not with an antibody against the unrelated protein cAMP response element-binding II (Figure 5A, lane 4 and 5). Similarly, incubation of rat aortic SMC extracts with the radiolabeled NF-κB consensus–binding site generated a retarded band that was competed with an excess of unlabeled consensus sequence, but not with an equivalent amount of the mutated sequence (Figure 5A, lane 6–8). This retarded band was also abrogated/supershifted using 2 anti-p65 antibodies, but not with anti-cAMP response element-binding II (Figure 5A, lane 9–11). These results demonstrate that murine aorta and aortic SMCs exhibit NF-κB DNA–binding activity. Importantly, we found higher NF-κB DNA–binding activity in mouse AA, and mouse and rat AA–SMCs than in their TA counterparts, although differences did not reach statistical significance for mouse AA and TA (Figure 5B and 5C). Accordingly, we observed higher expression of NF-κB targets in mouse AA and AA–SMCs than in their TA counterparts, examined by western blot analysis (Figure 5D) and flow cytometry (Figure 5E), respectively.

We next investigated whether HOXA9 regulates NF-κB activity in SMCs. For this analysis, SMCs were transiently

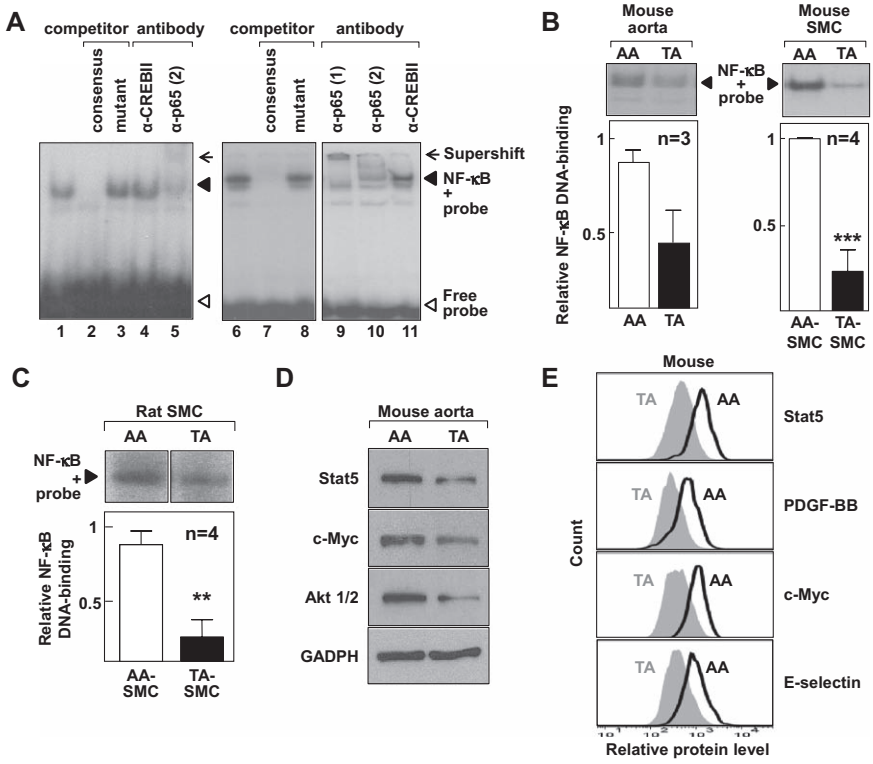

Figure 5. Nuclear factor-κB (NF-κB) DNA–binding activity and target gene expression are higher in aortic arch (AA) and AA–smooth muscle cells (SMCs) than in thoracic aorta (TA) and thoracic aorta–SMCs (TA–SMCs). **A**–**C**, Electrophoretic mobility shift assays (EMSA) using cell lysates and a radiolabeled oligonucleotide containing the NF-κB consensus DNA-binding site. Representative autoradiographs are shown, and quantitative data in **B** and **C** are mean±SEM of the indicated number of independent experiments. In the EMSA of each panel, all binding reactions were run in the same gel and exposed to the same X Ray film. Cell lysates in **A** were prepared from mouse TA (**left**) and tumor necrosis factor α-treated rat primary TA–SMCs (**right**). For competition assays, binding reactions included a 100-fold excess of unlabeled NF-κB consensus oligonucleotide or a mutant sequence that disrupts NF-κB binding. For supershift experiments, antibodies against p65 (1: sc-372; 2: sc-109) or the cAMP response element-binding (CREB II) (negative control) were added to binding reactions. Cell lysates in **B** and **C** were prepared from AA and TA, or from AA–SMCs and TA–SMCs isolated from wild-type mice or rat, as indicated. The autoradiographs in **B** and **C** only show the retarded nucleo-protein complexes. ***P*<0.01; ****P*<0.001. **D**, Western blot analysis of mouse AA and TA. **E**, Protein expression in AA–SMCs and TA–SMCs analyzed by flow cytometry.

transfected with the 5xNF-κB–luciferase reporter construct, in which luciferase expression is driven by a tandem repeat of 5 NF-κB–binding sites. Ectopic expression of human HOXA9, but not HOXA2, significantly reduced 5xNF-κB–luciferase activity in rat aortic E19P cells and, as expected, overexpression of the undegradable form of the NF-kappa-B inhibitor alpha (IκBα), which functions as a super-repressor of NF-κB,[28] reduced 5xNF-κB–luciferase activity (Figure 6A, top graph). Similar results were obtained in mouse and rat AA–SMCs (Figure 6A, middle and bottom graphs, respectively), which have higher NF-κB activity and lower *Hoxa9* expression than their TA counterparts. Thus, overexpression of HOXA9 significantly inhibits NF-κB–dependent transcription in rat aorta E19P cells and AA–SMCs.

We also examined whether NF-κB can inhibit transcription driven by the human HOXA9 promoter. Activity of the HOXA9 promoter–luciferase reporter was inhibited in E19P cells by cotransfection of NF-κB/p65 or treatment with tumor necrosis factor α (Figure 6B, top graph). The inhibitory effect of tumor necrosis factor α was abrogated by cotransfecting mut IκBα, demonstrating the involvement of NF-κB (Figure 6B, top graph). Similarly, NF-κB/p65 overexpression significantly inhibited *HOXA9* promoter–luciferase activity in mouse and rat TA–SMCs (Figure 6B, middle and bottom graphs, respectively), which have lower NF-κB activity and higher *Hoxa9* expression than their AA counterparts. Moreover, transfection with NF-κB/p65 or treatment with tumor necrosis factor α downregulated *Hoxa9* mRNA expression in rat E19P cells (Figure 6C).

To further examine the role of Hoxa9 in the regulation of NF-κB activity, we performed loss-of-function experiments in E19P and TA–SMCs. Cells were transiently transfected with small interfering RNA against *Hoxa9* (*Hoxa9*-siRNA) or control-siRNA, and protein expression was analyzed by flow cytometry. Compared with control-siRNA, *Hoxa9*-siRNA reduced the levels of endogenous Hoxa9 protein without affecting β-actin expression, and this was accompanied by increased expression of the NF-κB targets Stat 5, c-myc, and E-selectin (Figure 6D). Taken together, these results demonstrate higher NF-κB DNA–binding activity in athero-susceptible AA and AA–SMCs than in athero-resistant TA and TA–SMCs, and that HOXA9 and NF-κB inhibit each other in AA– and TA–SMCs isolated from wild-type mice and rats (Figure 6E).

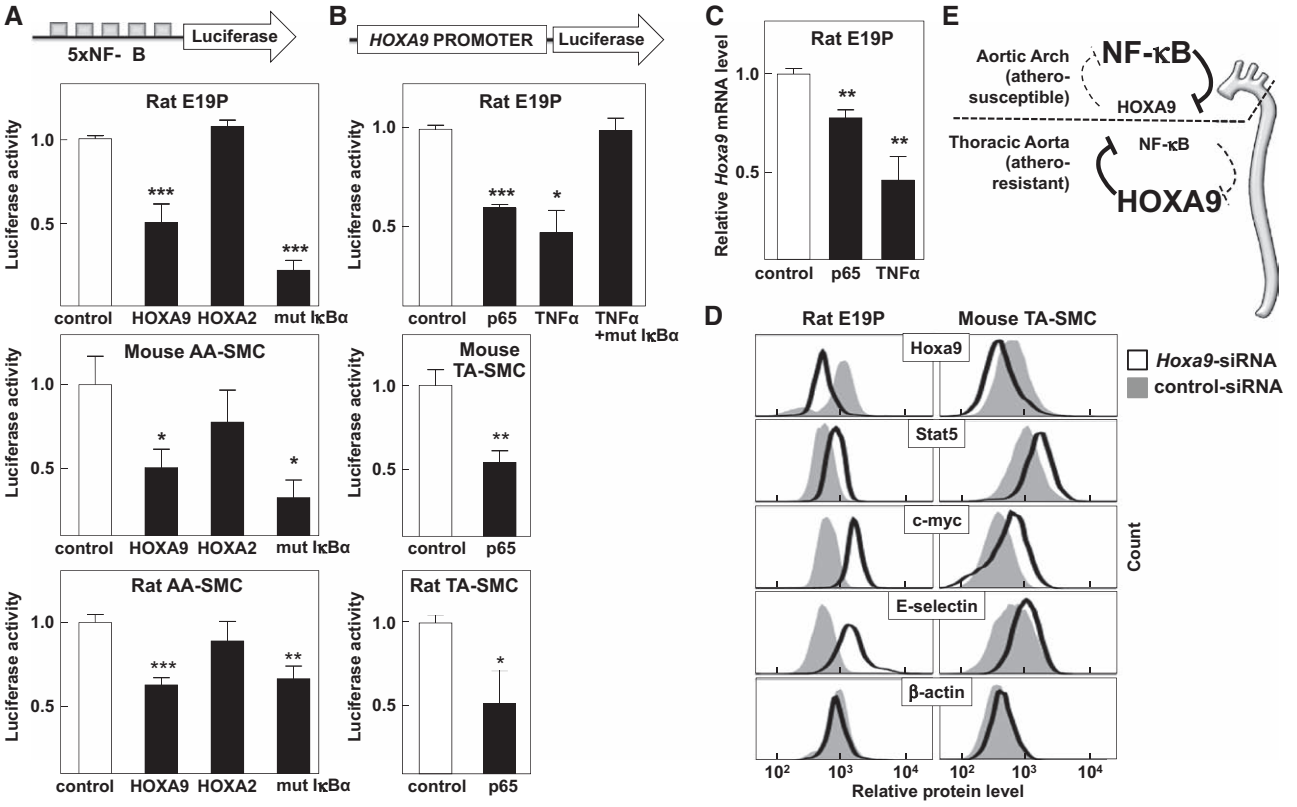

Figure 6. HOXA9 and nuclear factor-κB (NF-κB) inhibit each other in smooth muscle cells (SMCs). Experiments were performed with rat embryonic aortic E19P cells, and primary mouse or rat aortic arch (AA)– or thoracic aorta (TA)–SMCs, as indicated. **A**, Cells were transfected with a luciferase reporter gene containing a tandem repeat of 5 NF-κB–binding sites (5xNF-κB–luciferase) plus pRL-TK renilla as a control for transfection efficiency. When indicated, cells were cotransfected with HOXA9 (pcDNA3.1$^+$-HOXA9), HOXA2 (pcDNA3.1$^+$-HOXA2), or mut IκBα (IκBα S32/36A, NF-κB super–repressor). One day after transfection, cells were lysed, and luciferase activity was measured. Data are mean±SEM of 4 to 7 independent experiments. **B**, Cells were transfected with pGL3 basic–HOXA9 promoter–luciferase reporter plasmid plus pRL-TK renilla. When indicated, cells were cotransfected with mut IκBα or p65-GFP (subunit of NF-κB complex), and treated with tumor necrosis factor α (TNFα) (10 ng/mL during the last 4 hours). Luciferase activity was measured after 24 h. Data are mean±SEM of 3 to 6 independent experiments. **C**, E19P cells were untreated, exposed to TNFα (10 ng/mL, 4 h), or transiently transfected with p65-GFP, and total RNA was isolated for quantitative polymerase chain reaction (qPCR) to measure *Hoxa9* expression using 18S as an endogenous control. Results are mean±SEM of 4 independent experiments measured in triplicate (arbitrary units normalized to control cells). **D**, Cells were transiently transfected with small interfering RNA against Hoxa9 (*Hoxa9*-siRNA) or control-siRNA. Transfected cells were detached, and levels of the indicated proteins were measured by flow cytometry. **E**, Proposed model for the reciprocal modulation of NF-κB and HOXA9 in the adult aorta. *P<0.05; **P<0.01; ***P<0.001.

Discussion

The main objective of the present study was to identify factors involved in establishing vascular heterogeneity that may contribute to atherosclerosis development in adulthood. Our high-throughput microarray studies were carried out in young mice fed standard chow, in which atherosclerotic lesions were either absent (wild-type) or absent/very incipient (apoE-KO). This allowed us to detect genes differentially regulated in athero-prone AA and athero-resistant TA at prelesional stages, which may therefore be causally related to atherosclerosis development at later phases. Although we identified genes that were differentially expressed only in wild-type mice (27 genes) or only in apoE-KO mice (127 genes), we focused on the 219 genes that were altered in AA versus TA in both wild-type and apoE-KO mice, because these are more likely to be regulated by positional cues independently of genotype. Among these genes, *Hox* cluster genes 6 to 10 are highly expressed in mouse TA compared with AA. We also demonstrate that *Hox* genes are highly expressed in adult rat and swine TA compared with AA. This pattern of Hox-specified positional identity in the adult vasculature of mammals is also observed in developmental origin–specific human SMCs derived in vitro from embryonic stem cells and in mouse and rat primary SMCs isolated from adult AA and TA cultured under static conditions. We have also shown reciprocal negative regulation between HOXA9, which is highly expressed in TA, and the proinflammatory and proatherogenic transcription factor NF-κB, which is more active in AA. Based on these results in tissue and SMCs obtained from wild-type mice and rats, we propose that embryonically imprinted differential Hox expression may contribute to the establishment of distinct regional molecular signatures in the adult vasculature that are of likely importance in pathophysiological processes (Figure 6E).

Although our results in aortic tissue and SMC cultures are qualitatively consistent for most of the studied *Hox* genes (higher expression in TA and TA–SMCs than in AA and AA–SMCs), there are some dissimilarities in the size of the differences between aortic tissue and SMC cultures; for example, *Hoxa10* level is ≈10-fold higher in mouse TA than in AA, but ≈80-fold higher in TA–SMC than in AA–SMC (Figure 3A), and the *Hoxa9* level is >60-fold higher in rat TA than in AA, but only ≈8-fold higher in TA–SMC than in AA–SMC; Figure I in the online-only Data Supplement). These differences suggest that homeobox gene expression in SMCs might be affected by cues from other neighboring mural cell types, which might also exhibit AA versus TA differences in *Hox* gene expression. Therefore, it would be interesting to perform in situ hybridization experiments in AA and TA to identify the cell types expressing *Hox* genes, which might also reveal unknown gradients of *Hox* gene expression. To further explore the relationships between *Hox* expression and athero-susceptibility in different vascular cells, these in situ studies should also include other vessels. For example, it would be of interest to examine the athero-prone abdominal aorta, which contains SMCs of somitic-mesoderm origin, like the athero-resistant TA, and the athero-prone carotid artery, which contains SMCs from neural crest origin, like the athero-susceptible AA.[7,8,21]

The Hox transcription factors are known to be involved in determining positional identity and tissue specialization in the developing embryo in a wide range of evolutionarily distant animal species, including Caenorhabditis elegans, Amphioxus, Drosophila, fish, frog, chick, mouse, and humans.[17–20] Comprehensive expression profiling of highly homologous *Hox* genes in different postnatal and adult tissues suggests a continuation of embryonic patterning[29–31]; however, the functions of *Hox* genes after birth remain largely unexplored and subject to speculation.[31] Previous studies have implicated a few individual Hox and nonclustered homeobox genes in physiological and pathological cardiovascular remodeling.[32–34] For example, Miano et al[35] observed preferential *HOXB7* and *HOXC9* mRNA expression in fetal compared with adult SMCs and in rat pup compared with adult SMCs, consistent with the notion that differential *HOX* expression may contribute to establishing phenotypic diversity in these cells. Moreover, Chi et al[36] found that human SMCs isolated from different adult anatomic sites (bronchus, vein, artery, urinary tract, colon, uterus, cervix) can be clustered based solely on their pattern of expression of 63 homeobox genes, and suggested that positional information encoded in the pattern of *HOX* genes may play an important role in determining the distinct molecular phenotypes of SMCs from different organs. However, these authors did not compare differences in *HOX* gene expression between vascular SMCs obtained from different arteries, such as aorta and the coronary, pulmonary, iliac, and umbilical arteries. Indeed, information on the expression of *Hox* genes in different regions of the adult vasculature is very scarce, with only a few studies analyzing the expression of a limited number of individual family members and their correlation with susceptibility to vascular pathologies.[25,37–40] Endogenous gene expression studies and reporter gene analysis in transgenic mice revealed *Hoxa3* and *Hoxc11* expression in subsets of vascular SMCs and endothelial cells located in distinct regions of the adult vasculature that roughly corresponded to the embryonic expression domains of the 2 genes.[39] Higher HOXA1 expression and lower *HOXA9* and *HOXA10* expression were found in swine endothelium from athero-susceptible than from athero-resistant vessels.[25,37,40] Moreover, HOXA4 expression is lower in the adult baboon abdominal aorta than in the TA and in human abdominal aortic aneurysms than in control tissue.[38] As a first step toward understanding the intrinsic phenotypic differences between arterial segments with distinct susceptibility to atherosclerosis, we performed high-throughput transcriptomic analysis of mouse athero-resistant TA and atherosclerosis-prone AA. To our knowledge, our unbiased analysis of wild-type and apoE-KO mice is the first to identify a spatially coordinated and concerted expression of all members of the Hox paralogous groups 6 to 10 along the mature aorta, with higher expression of *Hox6-10* in TA than in AA. Importantly, we confirmed these results in TA and AA from adult rats and pigs. These findings are in agreement with the well-established notion of spatial colinearity of *Hox* genes during embryonic development, with the most downstream (3′) and upstream (5′) members of each cluster being expressed predominantly in anterior and posterior regions, respectively.[17–20]

The AA and TA are subject to different fluid mechanical forces generated by distinct blood flow patterns, and these forces make a central contribution to determining

atherosclerosis susceptibility at least in part by establishing specific genetic regulatory programs.[4–6] Therefore, differences in *Hox6-10* gene expression along the aorta might be imposed by hemodynamic factors. Although we find that the differences between AA and TA observed in vivo persist in primary AA–SMCs and TA–SMCs cultured under static conditions, we cannot rule out the possibility that blood flow–dependent irreversible epigenetic changes imprinted in vivo account for differential *Hox6-10* expression in cultured cells. Likewise, distinct paracrine effects caused by neighboring cells present in the vessel wall, including endothelial cells, fibroblasts, and immune cells, might impose irreversible epigenetic modifications on SMCs. This is improbable, however, given our studies using developmental origin–specific NE–SMCs and PM–SMCs derived from human embryonic stem cells, which express markers of differentiated SMCs and recapitulate key phenotypic features of AA–SMCs and TA–SMCs, respectively.[22] Although these in vitro–derived cells have never been exposed to fluid mechanical forces and were not cocultured with other cell types, we found higher *HOX6-10* expression in PM–SMCs than in NE–SMCs. These results support the idea that higher *Hox6-10* expression in TA is caused by hard-wired embryonic programs and not postnatal environmental cues.

Differential *Hox6-10* expression between the AA and TA might be a conserved mechanism that helps to explain why these vessel segments respond differently to common cardiovascular risk factors. However, demonstrating such a cause-and-effect relationship is difficult because of the involvement of multiple homeobox genes with a high degree of sequence similarity, particularly among paralogous *Hox* genes. Indeed, analysis of genetically modified mice with altered *Hox* expression has revealed extensive functional compensation, especially among *Hox* genes from the same paralogous group.[17] To begin to address the functional consequences of differential *Hox* gene expression in adult AA and TA, we focused on *Hoxa9*, which is implicated in endothelial cell migration, endothelial differentiation of adult progenitor cells, and postnatal neovascularization.[41,42] Moreover, endothelium from porcine and human athero-susceptible arteries exhibits comparatively low *HOXA9* mRNA expression and high NF-κB activity.[25,37,40] However, we are unaware of any earlier functional study of HOXA9 in SMCs, a major component of the arterial wall that plays a key role in vascular pathophysiology. Our results demonstrate that *Hoxa9* expression is lower in AA–SMCs and athero-susceptible AA than in TA–SMCs and athero-resistant TA, and this correlates with higher NF-κB activity and higher expression of NF-κB targets in AA–SMCs and AA. Moreover, in agreement with previous studies in endothelial cells,[27] our studies in SMCs show that overexpression of human HOXA9 inhibits NF-κB activity, which in turn represses reporter gene expression driven by the human HOXA9 promoter and rat *Hoxa9* mRNA expression. We have also shown that inhibition of Hoxa9 in TA–SMCs downregulates the expression of NF-κB target genes. These findings suggest that reciprocal inhibition between HOXA9 and NF-κB in endothelial cells and SMCs contributes to differences in athero-susceptibility between AA and TA (Figure 6E). Because our functional studies were carried out with SMCs obtained from wild-type rodents, future studies will be needed to ascertain whether this model can be applied in the setting of the chronic inflammatory stress that characterizes atherosclerosis progression. Further high-throughput studies are also warranted to identify the repertoire of genes differentially regulated in AA versus TA as a result of differential *Hox6-10* expression, which may shed light on the molecular mechanisms underlying the distinct response of vascular cells to cardiovascular risk factors depending on their embryonic origin.

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Disclosures

None.

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Significance

Different blood vessels and distinct segments within a given artery are heterogeneous in their susceptibility to developing vascular pathologies in response to common risk factors, but the molecular mechanisms underlying these differences are incompletely understood. Here, we show that homeobox cluster genes 6 to 10 are highly expressed in mouse athero–resistant thoracic aorta compared with athero-prone aortic arch. This pattern of Hox-specified positional identity is maintained in adult rat and swine thoracic aorta compared with aortic arch, in mouse and rat primary smooth muscle cells isolated from adult aortic arch and thoracic aorta, and in developmental origin–specific human smooth muscle cells derived in vitro from embryonic stem cells and cultured under static conditions. We also show that differential Hox expression contributes to maintaining phenotypic differences between smooth muscle cells from athero-resistant and athero-susceptible regions, at least in part through feedback regulatory mechanisms involving inflammatory mediators, for example, reciprocal inhibition between HOXA9 and NF-κB.