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Proteomic and Metabolomic Analysis of Smooth Muscle Cells Derived From the Arterial Media and Adventitial Progenitors of Apolipoprotein E–Deficient Mice

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Abstract—We have recently demonstrated that stem cell antigen 1–positive (Sca-1$^+$) progenitors exist in the vascular adventitia of apolipoprotein E–deficient (apoE$^{-/-}$) mice and contribute to smooth muscle cell (SMC) accumulation in vein graft atherosclerosis. Using a combined proteomic and metabolomic approach, we now characterize these local progenitors, which participate in the formation of native atherosclerotic lesions in chow-fed apoE$^{-/-}$ mice. Unlike Sca-1$^+$ progenitors from embryonic stem cells, the resident Sca-1$^+$ stem cell population from the vasculature acquired a mature aortic SMC phenotype after platelet-derived growth factor-BB stimulation. It shared proteomic and metabolomic characteristics of apoE$^{-/-}$ SMCs, which were clearly distinct from wild-type SMCs under normoxic and hypoxic conditions. Among the differentially expressed proteins were key enzymes in glucose metabolism, resulting in faster glucose consumption and a compensatory reduction in baseline interleukin-6 secretion. The latter was associated with a marked upregulation of insulin-like growth factor binding proteins (IGFBPs) 3 and 6. Notably, reconstitution of interleukin-6 to levels measured in the conditioned medium of wild-type SMCs attenuated the elevated IGFBP expression in apoE$^{-/-}$ SMCs and their vascular progenitors. This coregulation of apoE, interleukin-6, and IGFBPs was replicated in wild-type SMCs from hypercholesterolemic mice and confirmed by silencing apoE expression in SMCs from normocholesterolemic mice. In summary, we provide evidence that Sca-1$^+$ progenitors contribute to native atherosclerosis in apoE$^{-/-}$ mice, that apoE deficiency and hypercholesterolemia alter progenitor cell behavior, and that inflammatory cytokines such as interleukin-6 act as metabolic regulators in SMCs of hyperlipidemic mice. **(*Circ Res*. 2008;102:1046-1056.)**

Key Words: atherosclerosis ■ insulin-like growth factor-1 ■ progenitor cells ■ proteomics ■ vascular smooth muscle

With the introduction of apolipoprotein (apo)E-deficient strains, the mouse became the preferred animal model in cardiovascular research.[1] ApoE is a glycoprotein that is synthesized in the liver and the brain, but it is also produced locally in the vessel wall, mainly in infiltrating monocytes and macrophages,[2] and gets recruited from the circulation after vascular injury.[3] Besides apoE-mediated cholesterol transport, lipid-independent effects of apoE also have relevance in vitro and in vivo. For instance, apoE is synthesized in quiescent but not actively proliferating smooth muscle cells (SMCs) in culture[4] and suppresses growth factor and oxidized LDL–induced SMC migration and proliferation.[5] A possible role of apoE in modulation of SMC growth in vivo is supported by observations that the numbers of intimal SMCs are increased in fibroproliferative atherosclerotic plaques of chow-fed apoE$^{-/-}$ mice but reduced after vascular injury in transgenic mice overexpressing apoE.[1,6] Similarly, we found that vein grafts of apoE$^{-/-}$ mice showed increased neointima formation even if grafted to normolipidemic wild-type animals.[7] Notably, atherosclerosis is more severe in chow-fed apoE$^{-/-}$ mice than in cholesterol-fed apoE$^{+/+}$ mice despite similar plasma cholesterol levels.[8] Differences in protein expression and metabolism between apoE$^{-/-}$ and apoE$^{+/+}$ SMCs, however, remain to be elucidated.

Besides local SMCs, vascular progenitors may contribute to SMC accumulation in vascular disease.[9–14] We focused on a resident population of stem cell antigen 1 positive (Sca-1$^+$) progenitors present in the adventitia of apoE$^{-/-}$ mice that repopulates vein grafts following SMC death.[13,15–17] Although these progenitors express SMC markers on platelet-derived growth factor (PDGF)-BB stimulation,[13] a more comprehensive assessment at the molecular level is needed to establish whether these progenitor cell–derived cells truly belong to the SMC lineage. We have recently used proteom-

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From the Cardiovascular Division (M.M., A.Z., A.S., U.M., X.Y., Y.H., Q.X.), King's College, London; Department of Cardiac and Vascular Sciences (A.I.D.S.) and Department of Basic Medical Sciences (Y.-L.C.), St. George's, University of London, United Kingdom; Cancer Research UK Cambridge Research Institute (J.R.G., B.M.), United Kingdom; and Gaubius Laboratory and Leiden University Medical Center (P.H.Q.), The Netherlands.

Correspondence to Dr Manuel Mayr, Cardiovascular Division, The James Black Centre, King's College, University of London, 125 Coldharbour Ln, London SE5 9NU, United Kingdom. E-mail manuel.mayr@kcl.ac.uk

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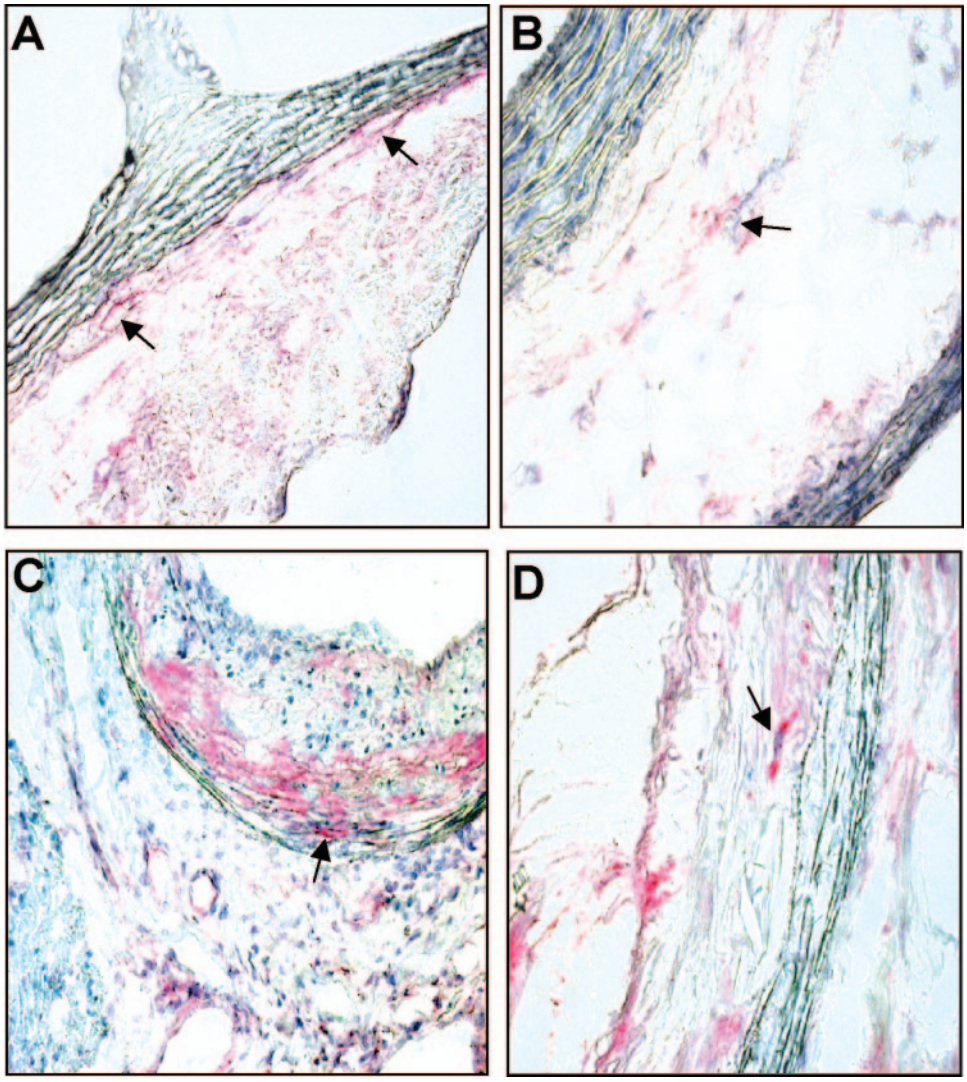

Figure 1. Sca-1$^+$ progenitors participate in atherosclerosis. Aortic roots from 10-week-old (A and B) and 12-month-old (C and D) apoE$^{-/-}$ mice were sectioned and labeled with anti–Sca-1 antibodies. Sections were developed with alkaline phosphatase anti–alkaline phosphatase (APAAP) techniques and counterstained with hematoxylin (blue). Arrows highlight Sca-1$^+$ cells. Note that Sca-1$^+$ cells are predominantly detectable in early (C) rather than complex (D) atherosclerotic lesions.

ics to demonstrate that Sca-1$^+$ progenitors derived from embryonic stem cells can express a panel of SMC markers in response to PDGF-BB stimulation without acquiring a mature SMC phenotype.[18] Instead, these SMC-like cells maintained characteristics of their embryonic stem cell origin.[19] Consequently, the question arose as to whether SMCs derived from adult progenitor cells in the adventitia of apoE$^{-/-}$ mice would be more similar to mature aortic SMCs. Because the phenotype of progenitor–derived cells is better reflected in their instantaneous protein profiles than the expression of a selected panel of marker proteins,[20] we compared the proteome of SMCs derived from adult Sca-1$^+$ progenitors with aortic SMCs derived from apoE$^{-/-}$ and apoE$^{+/+}$ mice by using difference in-gel electrophoresis (DIGE) and tandem mass spectrometry.

Materials and Methods

An expanded Materials and Methods section is available in the online data supplement http://circres.ahajournals.org.

Key techniques involved adaptations of previously published protocols, including those for the culture of SMCs,[21] the isolation of Sca-1$^+$ progenitors from the aortic adventitia,[13] differentiation of embryonic stem cells into SMC-like cells,[18] immunohistochemistry,[7] 2D gel electrophoresis,[22] tandem mass spectrometry,[23] NMR spectroscopy,[23] and RNase Protection assay.[24] Protocols for proteomic analysis are available on our web site at http://www.vascular-proteomics.com.

Results

Resident Sca-1$^+$ cells contribute to atherogenesis in apoE$^{-/-}$ mice. Sca-1$^+$ cells resided within the adventitia of aortas from 10-week-old apoE$^{-/-}$ mice (Figure 1A and 1B) but not apoE$^{+/+}$ mice.[13] Their recruitment to the vasculature coincided with increased expression of the CXC chemokine stromal cell–derived factor (SDF)-1α[25]: real-time PCR measurements revealed that the SDF-1α/GAPDH ratio was 0.97±0.12 and 0.74±0.10 in aortas of apoE$^{-/-}$ and apoE$^{+/+}$ mice, respectively (n=4, P=0.028). On cholesterol feeding, Sca-1$^+$ cells also appeared in the

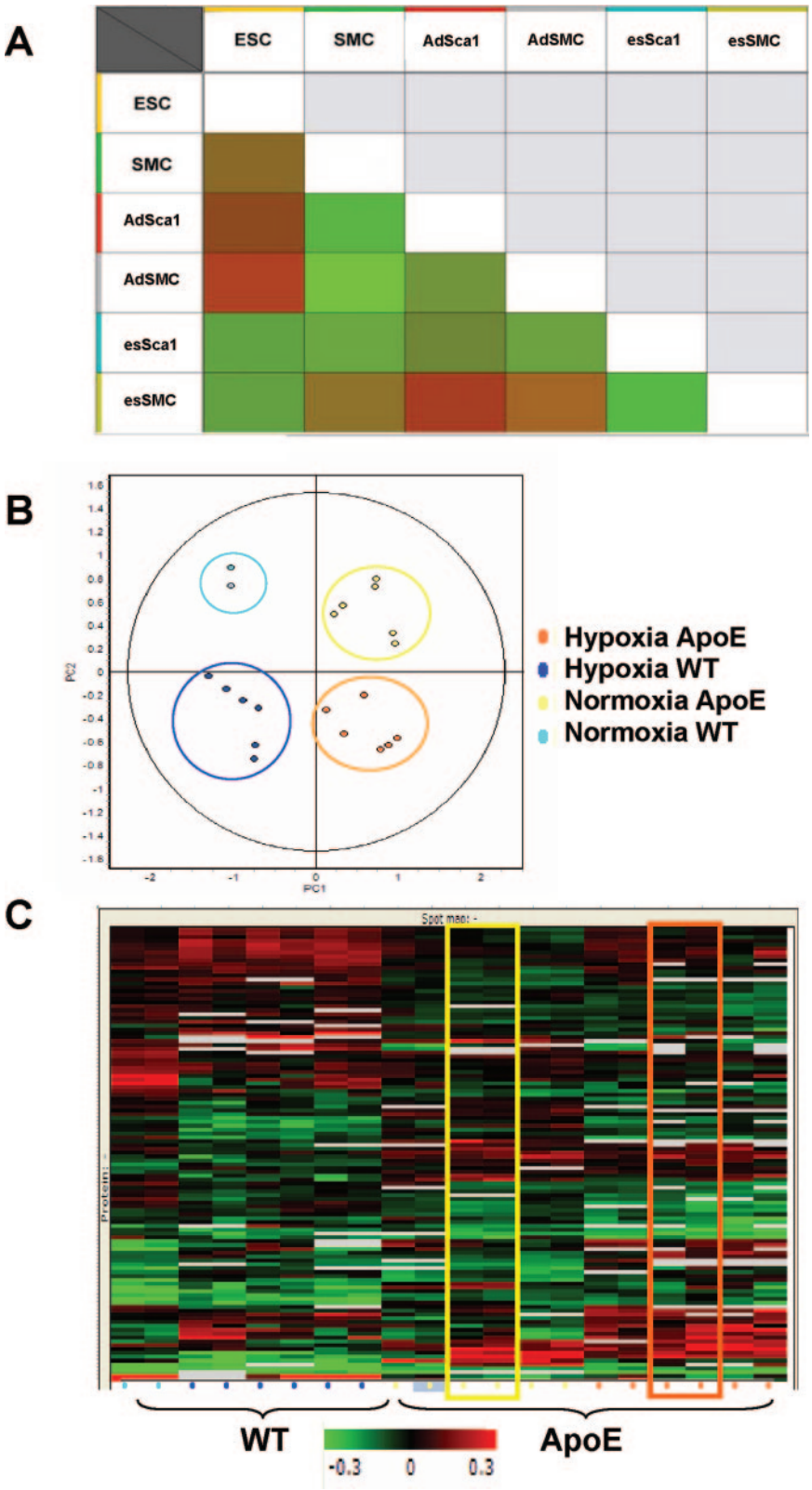

Figure 2. Proteomic characterization of SMCs and their vascular progenitors. A, Match matrix highlighting similarity between proteomic profiles of Sca-1$^+$ progenitors derived from embryonic stem cells or isolated from the adventitia of adult mice before and after PDGF-BB treatment. Bright green color denotes similarity; brown and red color, dissimilarity. Note that under PDGF-BB treatment, Sca1$^+$ progenitors from adult mice, but not from embryonic stem cells, resemble a mature SMC phenotype. ESC indicates embryonic stem cells; SMC, mature aortic SMCs; AdSca1, Sca-1$^+$ progenitors derived from the adventitia; AdSMC, adventitial Sca-1$^+$ progenitors after PDGF-BB stimulation; esSca1$^+$, Sca-1$^+$ progenitors derived from murine embryonic stem cells; esSMCs, embryonic stem cell–derived Sca-1$^+$ progenitors after PDGF-BB stimulation. The score plot in B shows a principal component analysis of proteomic profiles from SMCs derived from adult Sca-1$^+$ progenitors and from aortic SMCs of apoE$^{+/+}$ (Wt) and apoE$^{-/-}$ (apoE) mice under normoxic and hypoxic conditions. The black ellipse represents the 95% significance level. C, Hierarchical clustering was applied to rearrange the dataset. The spot maps in each experimental group were divided into 4 clusters. All apoE$^{+/+}$ and apoE$^{-/-}$ SMCs were closely grouped. Furthermore, there was a clear separation between hypoxia and normoxia. Moreover, SMCs derived from adventitial Sca1$^+$ progenitors (boxed lanes) were grouped with apoE$^{-/-}$ SMCs. X-axis indicates spot maps; y-axis, proteins. Red color denotes increase; green color, decrease. Black color indicates no change. Bar shows the log standard abundance value interval for the colors; $\pm$0.3 denotes proteins with 3-fold increase or decrease.

adventitia of apoE$^{+/+}$ mice but fewer compared with apoE$^{-/-}$ mice (data not shown). During atherogenesis, numerous Sca-1$^+$ progenitors were present in early lesions (Figure 1C), whereas they were scarce in complex atheroma of 12-month-old apoE$^{-/-}$ mice (Figure 1D). Nonetheless, even in advanced lesions, there were almost twice as many cells staining for Sca-1 ($\approx$2.3$\pm$0.9% of total lesional cells of the aortic root) compared with other progenitor cell markers, including c-kit (1.1$\pm$0.3%), CD34 (1.6$\pm$0.5%), and fetal liver kinase 1 (0.4$\pm$0.3%). No staining was observed for the embryonic stem cell marker SSEA-1 (data not shown).

Proteomic Characterization of Progenitor Cell–Derived SMCs

To get a better understanding of their biological potential, Sca-1$^+$ resident progenitor cells were isolated from the adventitia as described previously[13] and characterized by proteomics. For comparison, Sca-1$^+$ progenitors were also prepared from embryonic stem cells.[18,26] As indicated by the match matrix of their proteomic profiles (Figure 2A), the similarity of Sca-1$^+$ progenitors to mature aortic SMCs was independent of their vascular or embryonic origin. Unlike Sca-1$^+$ progenitors derived from embryonic stem cells, however, the proteome of adventitial Sca-1$^+$ cells closely resembled mature SMCs after PDGF-BB treatment. To accurately quantify differences in protein expression, we compared apoE$^{+/+}$, apoE$^{-/-}$, and Sca-1$^+$–derived SMCs under normoxia and hypoxia (18 hours, 5% O_2 balanced with N_2) using DIGE. Principal component analysis (Figure 2B) and hierarchical clustering (Figure 2C) revealed that after in vitro differentiation with PDGF-BB, the proteome of adult Sca-1$^+$ progenitors was reminiscent of their apoE$^{-/-}$ origin.

Proteomic Comparison of ApoE$^{-/-}$ SMCs Under Normoxia

Among the differentially expressed proteins (n=6, supplemental Figure I and supplemental Table I) were chaperones and endoplasmic reticulum proteins of the unfolded protein response quality-control system (UPR), such as erp29/Bip, glucose-regulated protein 78 and 94 (grp78, grp94), and protein disulfide isomerases A3 and A6. The UPR system has previously been shown to be activated at all stages of atherosclerosis in apoE$^{-/-}$ mice.[27] It reduces new protein synthesis by translational attenuation and eliminates misfolded proteins by the ubiquitin proteasome system. This is consistent with the observed downregulation of enzymes involved in amino acid metabolism and eukaryotic elongation factors, eEF1 delta and eEF2. The latter mediates the translocation step of elongation and is phosphorylated by a calcium- and calmodulin-dependent protein kinase regulated by insulin through the rapamycin-sensitive mTOR pathway.[28] When the differentially expressed proteins (n=51) were submitted to Ingenuity Pathway Analysis (Ingenuity System, Mountain View, Calif), the computational algorithms built 3 protein association networks (supplemental Figure II) and returned downregulation of amino acid metabolism and upregulation of glycolysis/glucose metabolism as the top canonical pathways.

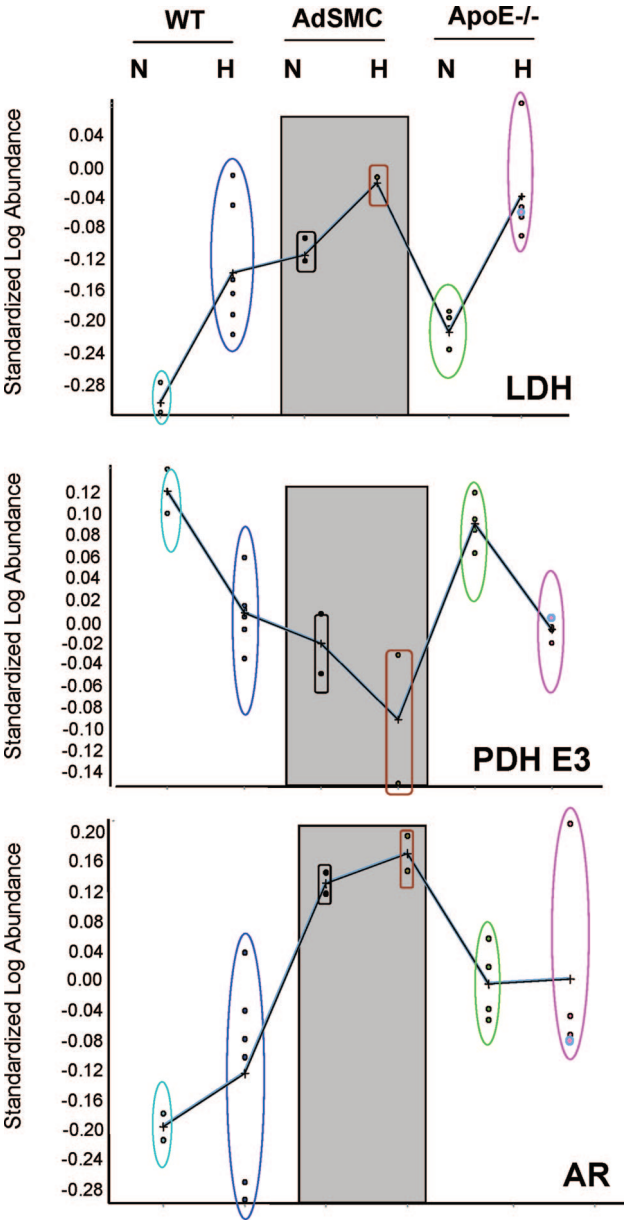

Figure 3. Representative enzymatic changes. Key enzymes in glucose metabolism were differentially expressed in wild-type (Wt), apoE$^{-/-}$, and adventitial progenitor-derived SMCs (AdSMC) under normoxic (N) and hypoxic (H) conditions. Note that the pattern for lactate dehydrogenase (LDH), dihydrolipoyl dehydrogenase (PDH E3), and aldose reductase (AR) in AdSMCs is more similar to mature apoE$^{-/-}$ SMCs than wild-type controls.

Proteomic Comparison of ApoE$^{-/-}$ SMCs Under Hypoxia

Protein changes in hypoxic (n=12) compared with normoxic (n=8) SMCs are summarized in supplemental Figure III and supplemental Table II. In all cell lines, hypoxia induced an upregulation of glycolytic enzymes, as well as lactate dehydrogenase, with a concomitant downregulation of the pyruvate dehydrogenase complex, the bridge between aerobic and anaerobic glucose metabolism (supplemental Figure IV). Key enzymes of alternative glucose pathways, however, were predominantly upregulated in mature and progenitor-derived apoE$^{-/-}$ SMCs, ie, aldose reductase for the sorbitol pathway (Figure 3). The metabolic alterations in apoE-deficient SMCs were associated with an increase of transaldolase, the reversible link between glycolysis and the pentose phosphate pathway, as well as isoforms of cytosolic malate dehydrogenase and aspartate aminotransferase, the 2 enzymatic components of the malate-aspartate shuttle, which is responsible for transporting reducing equivalents from glycolysis into mitochondria. In addition, several of the differentially expressed proteins identified in normoxic apoE$^{-/-}$ SMCs were confirmed after hypoxia and their quantitative differences were almost identical in the 2 independent proteomic datasets, eg, for ezrin, fascin, annexin A2, and eEF2 (supplemental Figure

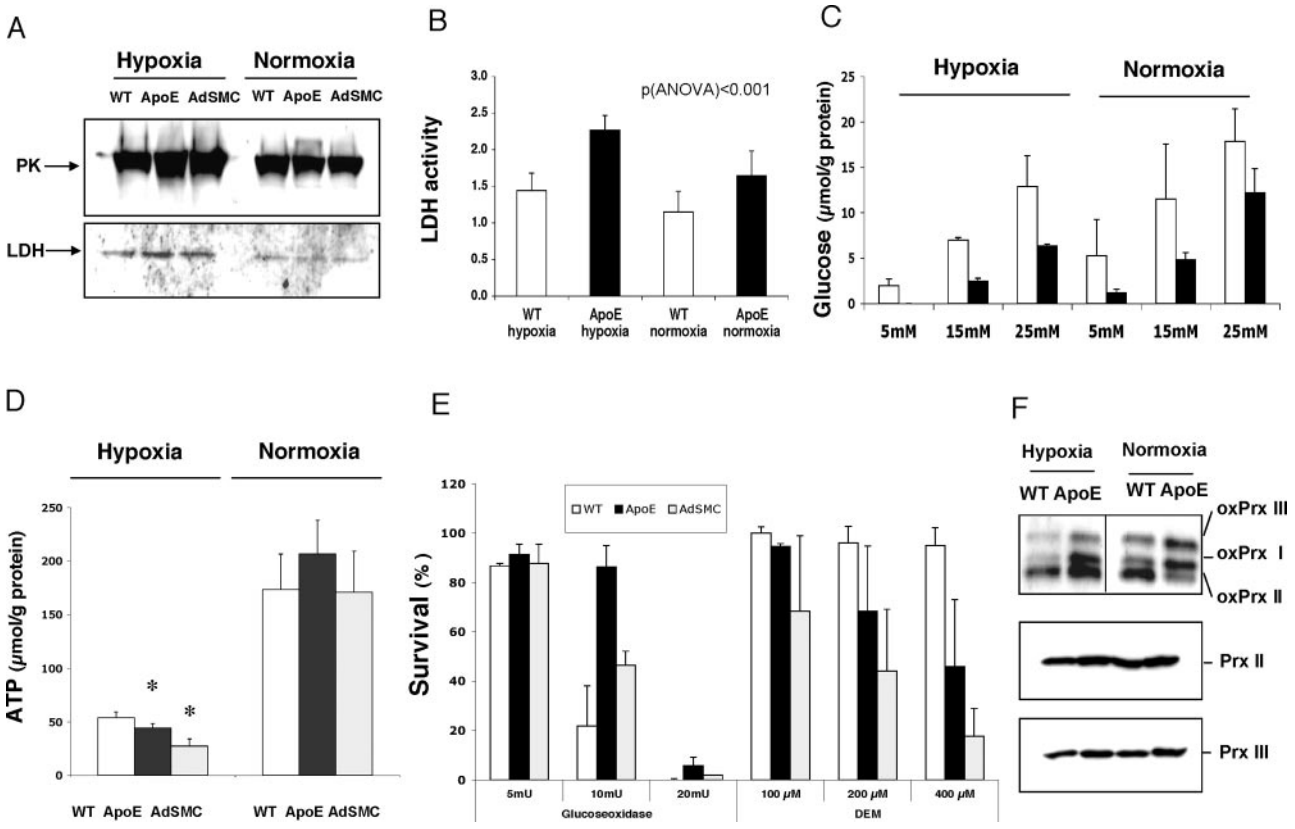

Figure 4. Glucose metabolism. A, Protein extracts of wild-type (Wt) and apoE$^{-/-}$ SMCs were probed with antibodies to pyruvate kinase (PK) and lactate dehydrogenase (LDH). B through D, Lactate dehydrogenase activity (B), glucose depletion in the culture medium (C), and cellular ATP (D), as measured under normoxic and hypoxic conditions. E, Increased susceptibility to oxidative stress–induced cell death. Survival of wild-type (white bars), apoE$^{-/-}$ SMCs (black bars), and progenitor-derived SMCs (gray bar) after incubation with glucose oxidase or diethylmaleate (DEM) in high glucose medium (25 mmol/L) for 24 hours is shown. Significant difference from controls: *$P<0.05$, **$P<0.01$. F, Oxidation of redox-sensitive proteins. Peroxiredoxins are a family of antioxidants that act by being the reducing substrate itself. Differences in the sulfoxidation of cytosolic (I, II) and mitochondrial (III) peroxiredoxins, as quantified by specific antibodies recognizing only the oxidized isoforms. No difference was observed for total Prx II and III.

V and supplemental Table III). Thus, cellular differences persisted in hypoxia, but enzymatic changes became more pronounced.

Immunoblotting and Enzymatic Assays

Consistent with our proteomic experiment, pyruvate kinase and lactate dehydrogenase increased in both apoE$^{+/+}$ and apoE$^{-/-}$ SMCs after hypoxia (Figure 4A), with the latter showing a tendency toward even higher levels. Despite similar net enzyme concentrations under normoxia, lactate dehydrogenase activity (Figure 4B) and glucose consumption (Figure 4C) were elevated in apoE$^{-/-}$ SMCs. ATP levels were similar to apoE$^{+/+}$ SMCs under normoxic conditions but lower in apoE$^{-/-}$ SMCs and progenitor-derived SMCs after hypoxia (Figure 4D). Compared with wild-type controls, apoE$^{-/-}$ SMCs and their progenitors were resistant to treatment with glucose oxidase as substrate depletion in the culture medium protected them against glucose oxidase–mediated oxidative injury (Figure 4E). They were, however, more susceptible to cell death in response to other oxidative stress stimuli,[7] ie, lowering the intracellular antioxidant glutathione by treatment with diethylmaleate (Figure 4E), and increased free radical generation in apoE$^{-/-}$ SMCs was evidenced by oxidation of redox-sensitive proteins, such as peroxiredoxins, providing additional confirmation of our proteomic data (supplemental Table III and Figure 4F).

Metabolomic Comparison of apoE$^{-/-}$ SMCs

To further clarify the metabolic effects of apoE deficiency, we measured metabolites in cellular extracts of normoxic SMCs cultivated in normal (5 mmol/L) and high glucose concentrations (25 mmol/L) by high-resolution NMR spectroscopy.[23] Quantitative data are included as supplemental Table IV. High glucose concentrations (25 mmol/L) resulted in a rise of myoinositol, which is exchanged for sorbitol to maintain osmoregulation. Notably, lactate levels were higher in apoE$^{-/-}$ than apoE$^{+/+}$ SMCs, and carnitine, required for the import of long-chain fatty acids into mitochondria and for transporting acetyl-coenzyme A out of the mitochondria to avoid a fatty acid–induced block of glycolysis, decreased by normalizing glucose concentrations in the culture medium in apoE$^{-/-}$, but not apoE$^{+/+}$ SMCs.

The effect of hypoxia on SMC metabolites is summarized in supplemental Table V. Apart from glycolic acid, cellular metabolites were similar in hypoxic apoE$^{+/+}$ and apoE$^{-/-}$ SMCs. NMR spectroscopy, however, confirmed that apoE$^{-/-}$ SMCs consumed glucose faster than their wild-type controls, as indicated by a depletion of glucose in the conditioned

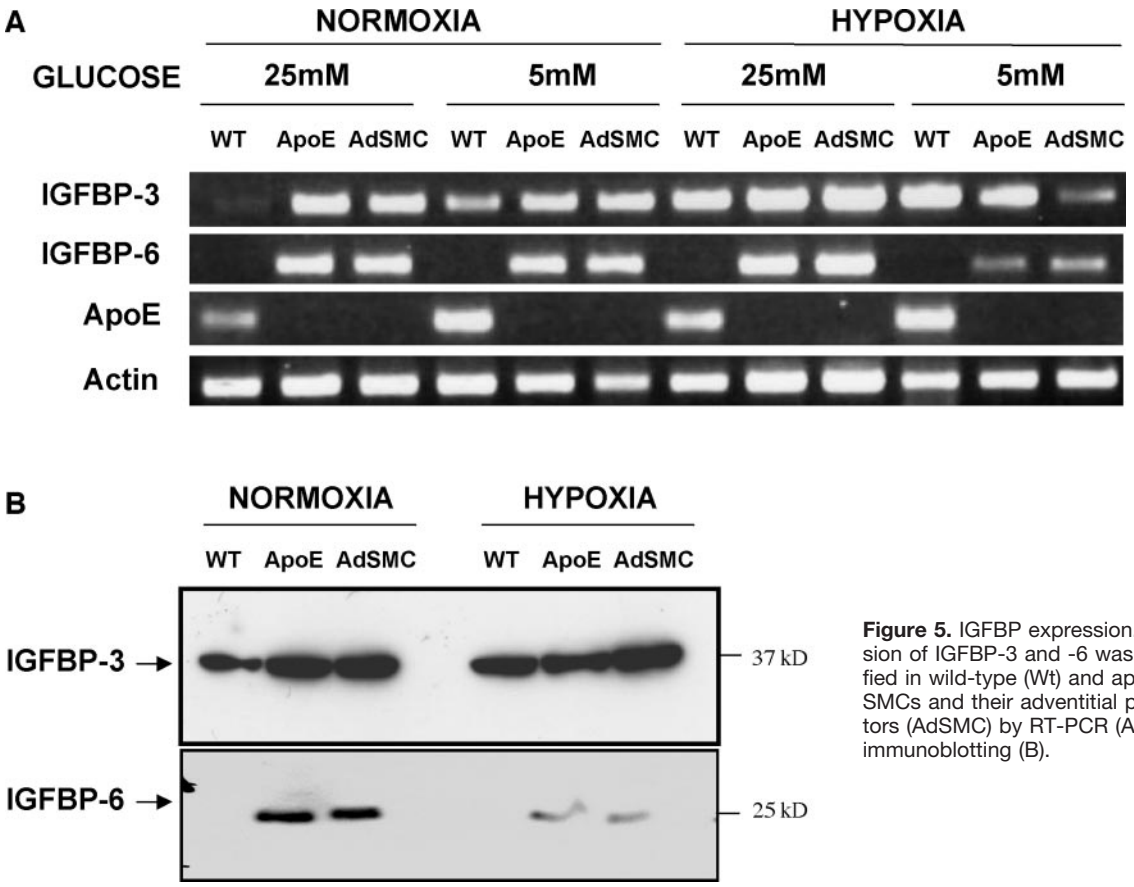

Figure 5. IGFBP expression. Expression of IGFBP-3 and -6 was quantified in wild-type (Wt) and apoE$^{-/-}$ SMCs and their adventitial progenitors (AdSMC) by RT-PCR (A) and immunoblotting (B).

medium and a corresponding rise in acetate, an endproduct of lipid metabolites (supplemental Figure VI). Thus, metabolism in apoE$^{-/-}$ SMCs was similar to hypoxic but not normoxic SMCs.

Interleukin-6 and Insulin-Like Growth Factor Binding Proteins

Apart from systemic factors, such as insulin, glucose metabolism is also regulated locally, ie, by the production of insulin-like growth factor (IGF) binding proteins (IGFBPs). We, therefore, evaluated IGFBP-1 to -7 expression in apoE$^{+/+}$, apoE$^{-/-}$, and progenitor-derived SMCs using RT-PCR. Whereas IGFBP-1 and -5 were undetectable in murine SMCs, expression of IGFBP-4 and -7 was identical in all cell lines. ApoE deficiency, however, was associated with a marked increase in IGFBP-3 and -6 at the mRNA (Figure 5A) and at the protein level (Figure 5B). Notably, IGFBP-3 was induced in apoE$^{+/+}$ SMCs by lowering glucose or oxygen concentrations in the culture medium, whereas IGFBP-6 was not inducible in wild-type SMCs despite its abundance in apoE$^{-/-}$ and progenitor-derived SMCs.

It has been demonstrated previously that tumor necrosis factor (TNF)-α regulates IGFBP-3 expression in SMCs.[29] Because TNF-α secretion was below detectable levels in the culture supernatant of murine SMCs (data not shown), we used a global approach (ie, RNase protection assay) to compare the capacity of wild-type, apoE$^{-/-}$, and progenitor-derived SMCs to produce other inflammatory mediators, including interleukin (IL)-1α, IL-1β, IL-1Ra, IL-6, IL-10, IL-12p35, IL12p40, IL-18, interferon-γ, and macrophage migration inhibitory factor. Expression was compared at baseline and after stimulation with bacterial lipopolysaccharides or TNF-α because apoE has been shown to suppress type I inflammatory responses in vivo.[30] Representative gels are shown in Figure 6. Whereas a transcriptional upregulation of interferon-inducible protein-10 and T-cell activation gene 3 was observed in apoE$^{-/-}$ SMCs and progenitor-derived SMCs after treatment with TNF-α and lipopolysaccharides, baseline mRNA transcripts for IL-18 were higher and for IL-6 lower in apoE$^{-/-}$ SMCs compared with wild-type controls. Subsequent measurements at the protein level confirmed a marked reduction of IL-6 secretion (Figure 7A, mean$\pm$SEM: 1.0$\pm$0.2 ng/mL in apoE$^{-/-}$ SMCs and 4.8$\pm$1.7 ng/mL in progenitor-derived SMCs versus 24.3$\pm$1.8 ng/mL in apoE$^{+/+}$ SMCs, respectively, $P<0.001$ [ANOVA]). Whereas glucose concentrations in the culture medium did not alter the baseline levels of IL-6 secretion in normoxic apoE$^{-/-}$ SMCs, the hypoxia-induced increase of IL-6 in apoE$^{-/-}$ SMCs was more pronounced in normal (5 mmol/L) than high (25 mmol/L) glucose medium (4.8 ng/mL versus 2.7 ng/mL, paired t test $P<0.05$). In contrast, glucose concentrations had no significant effect on the IL-6 release from hypoxic wild-type SMCs (data not shown). Thus, IL-6 secretion is substantially lower in apoE$^{-/-}$ SMCs but more responsive to glucose concentrations under hypoxia.

Next, we evaluated whether IL-6 would alter the IGFBP system. SMCs were incubated in fresh culture medium supplemented with 1 ng/mL and 10 ng/mL IL-6 for 24 hours.

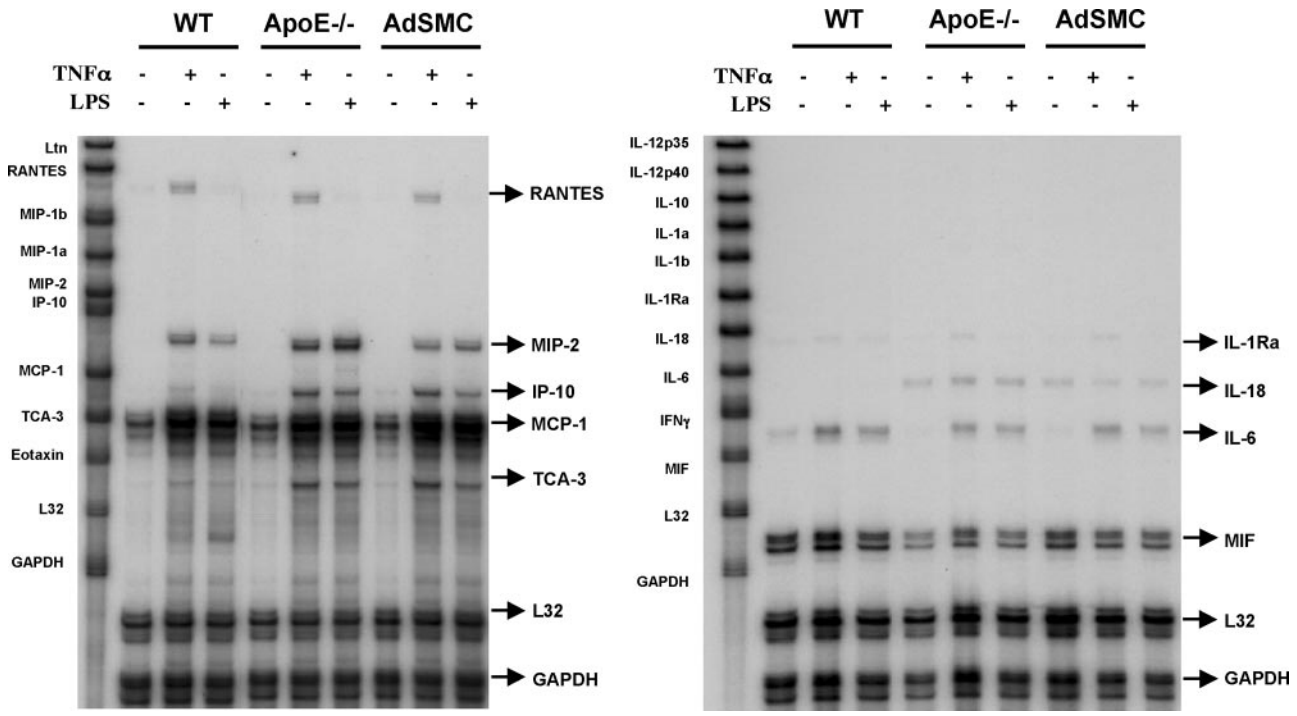

Figure 6. Cytokine expression. Total RNA was isolated from cells and analyzed by RNase Protection assay. GAPDH was used as a loading control.

Subsequently, mRNA transcripts for IGFBPs were quantified by RT-PCR. The administration of IL-6 resulted in transcriptional downregulation of IGFBP-3 and -6 but not other IGFBPs in apoE$^{-/-}$ and progenitor-derived SMCs (Figure 7B), demonstrating that the reduced levels of IL-6 account for the elevated IGFBP expression in apoE$^{-/-}$ SMCs.

IGFBPs in ApoE$^{+/+}$ SMCs From Hypercholesterolemic Mice

To establish whether the observed alterations resulted from apoE deficiency or hypercholesterolemia, we isolated aortic SMCs from cholesterol-fed wild-type mice. In these cells, too, a downregulation of apoE and IL-6 was associated with an upregulation of IGFBP-3 and IGFBP-6, confirming our concept of coregulation of apoE, IL-6 and IGFBPs (Figure 8A and 8B). Next, we silenced apoE expression using small interfering (si)RNA technology: downregulation of apoE by RNA interference was sufficient to reduce IL-6 secretion and to elevate IGFBP-6 expression in SMCs from normocholesterolemic mice. IGFBP-3, however, was not affected (Figure 8A and 8C). Thus, the observed phenotype in apoE$^{-/-}$ SMCs is probably the result of a combined effect of apoE deficiency and hypercholesterolemia.

Discussion

Possible roles of local progenitor cell populations within the vessel wall include a physical reconstruction of tissue during vascular repair, a paracrine support for growth of endogenous cells, or a limitation of inflammation. In the present study, we demonstrate that resident Sca-1^{+} cells migrate from the adventitia to the media during early atherosclerosis until they finally blend into the tissue in more complex lesions. Using a proteomic approach, we established that these adult progenitors have the potential of acquiring a mature SMC phenotype: unlike Sca-1^{+} progenitors derived from embryonic stem cells, Sca-1^{+} cells from the adventitia of aortas of apoE$^{-/-}$ mice had protein profiles similar to aortic SMCs after incubation with PDGF-BB in vitro. Notably, they maintained functional alterations indicative of their apoE$^{-/-}$ origin, as evidenced by their accelerated glucose consumption, increased transcription of IL-18, decreased synthesis of IL-6, elevated expression of IGFBP-3 and -6, and their susceptibility to oxidative stress. Thus, our data support the possibility of a physical incorporation of adult progenitors in the vasculature, although their overall similarity to mature apoE$^{-/-}$ SMCs, especially with respect to cytokine profiles, argues against a paracrine support or a limiting effect on inflammation.

Metabolism in ApoE$^{-/-}$ SMCs

In our previous proteomic and metabolomic analysis of aortas from apoE$^{-/-}$ mice,[31] we demonstrated that inefficient energy metabolism and increased oxidative stress preceded atherosclerotic lesion formation in hyperlipidemic animals. It is well established that oxygen consumption in the vasculature is augmented by accumulation of inflammatory cells and that lipid deposition in the arteries reduces the diffusion distance of oxygen and water soluble metabolites such as glucose, the main source of energy for the vasculature. Interestingly, cultivated aortic SMCs from apoE$^{-/-}$ mice showed accelerated glucose consumption and increased susceptibility to oxidative stress. Notably, the hyperglycemia-induced downregulation of the glucose transport is important for protecting cells against an excessive influx of glucose, a key factor for oxidative stress.

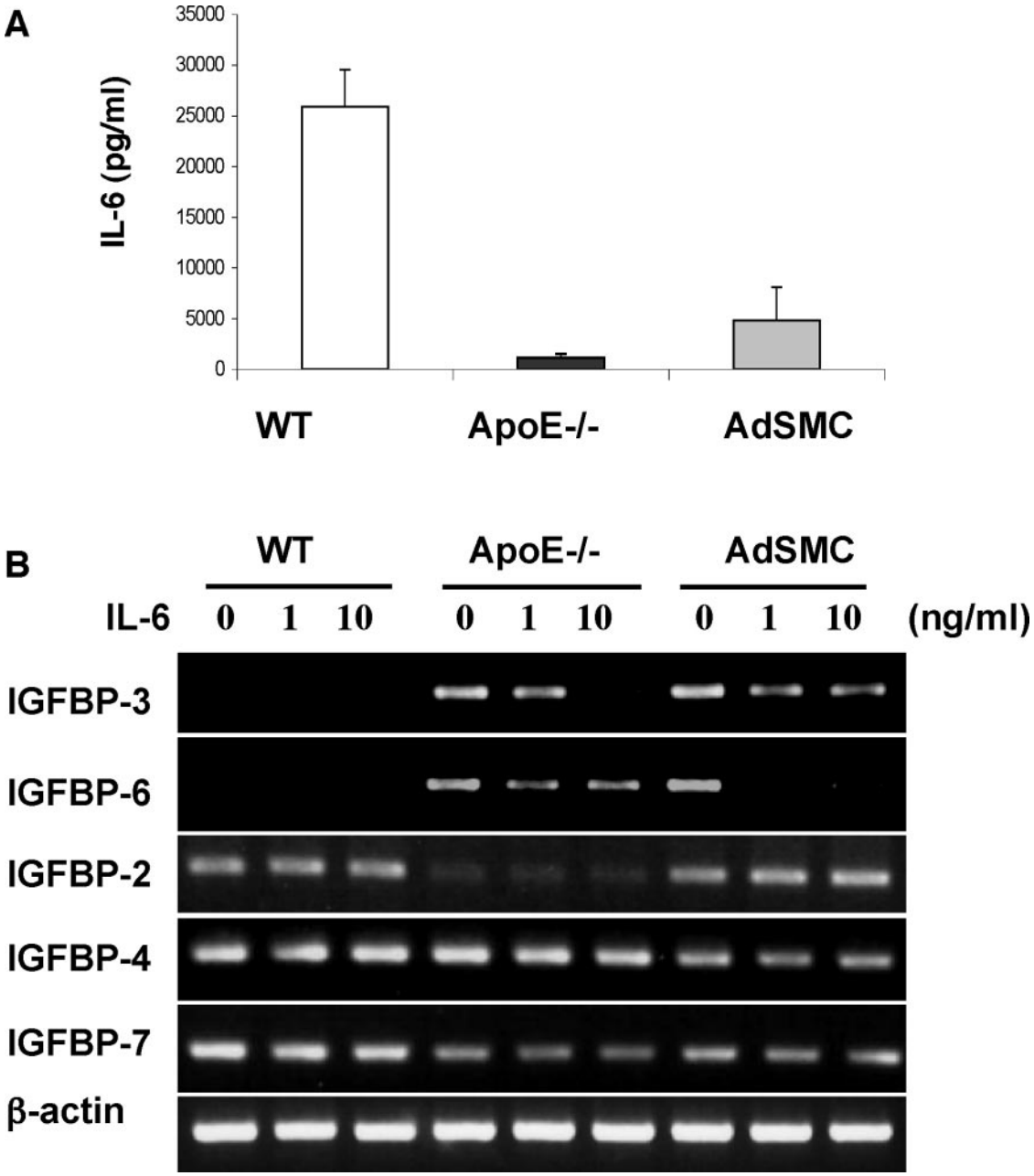

Figure 7. IL-6 attenuates IGFBP expression. A, IL-6 concentration in the conditioned medium. B, Effect of IL-6 supplementation on IGFBP expression.

Insulin-Like Growth Factor Binding Proteins

Glucose metabolism is modulated by insulin sensitivity in the target tissue. IGF-1 is a potent hypoglycemic agent with a similar function to insulin and an important survival factor for SMCs in atherosclerotic lesions.[32,33] Unlike insulin, the bioavailability of IGF in the circulation and in the extracellular space is regulated by IGFBPs, which are produced by local tissues and modulate IGF-1 effects.[32] Whereas liver-derived IGFBP-1 is the main binding partner for IGF-1 in the circulation and IGFBP-1 circulating levels correlate negatively with cardiovascular disease, IGFBP-3 has a similar ability to bind IGF-1 but is also produced in local tissues and plasma levels increase in patients with cardiovascular disease.[34] Notably, IGFBP-3 has intrinsic bioactivity and has recently been implicated in the differentiation of hematopoietic progenitors during vascular development.[35] In this respect, it is noteworthy that IL-6 supplementation normalized IGFBP-3 levels faster than IGFBP-6 in progenitor-derived SMCs, whereas the opposite was the case in mature apoE$^{-/-}$ SMCs (Figure 7B).

The expression, secretion, and regulation of IGFBPs in SMCs are known to be species specific: rat and murine wild-type SMCs expressed predominantly mRNA transcripts for IGFBP-2 and 4.[36] Whereas angiotensin II, thrombin, and reactive oxygen species reduced levels of IGFBP-4, IGFBP-2 and IGFBP-4 significantly increased in response native LDL and oxidized LDL and TNF-α induced IGFBP-3.[29,32,36] The present study is, to our knowledge, the first evidence that

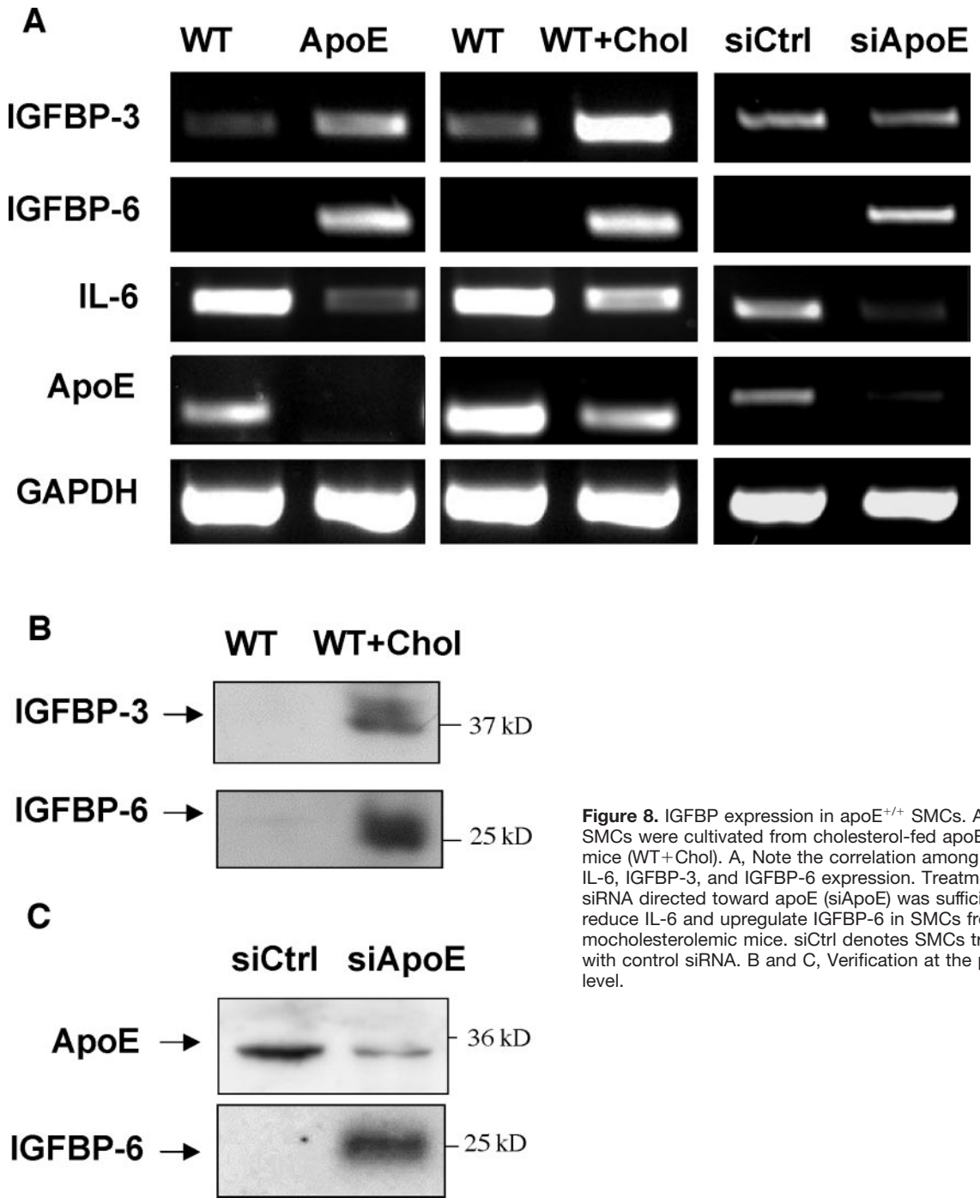

Figure 8. IGFBP expression in apoE$^{+/+}$ SMCs. Aortic SMCs were cultivated from cholesterol-fed apoE$^{+/+}$ mice (WT+Chol). A, Note the correlation among apoE, IL-6, IGFBP-3, and IGFBP-6 expression. Treatment with siRNA directed toward apoE (siApoE) was sufficient to reduce IL-6 and upregulate IGFBP-6 in SMCs from normocholesterolemic mice. siCtrl denotes SMCs treated with control siRNA. B and C, Verification at the protein level.

glucose depletion and hypoxia stimulate the expression of IGFBP-3, whereas the same stimuli had no effect on IGFBP-6. Both, IGFBP-3 and IGFBP-6, however, were upregulated in apoE$^{-/-}$ SMCs and progenitor-derived SMCs. This phenotype was replicated in apoE$^{+/+}$ SMCs from cholesterol-fed mice. To exclude that our findings were attributable to an inflammatory response following chronic hypercholesterolemia, we silenced apoE expression in wild-type SMCs from normocholesterolemic mice: in this experiment, too, downregulation of apoE was accompanied by decreased IL-6 production and a rise in IGFBP-6. Only IGFBP-3 was unchanged. These findings are consistent with our observations that IGFBP-3 is more responsive to environmental factors, ie, glucose and oxygen concentrations in the culture medium, and less dependent on the expression levels of apoE than IGFBP-6 (Figure 5). As summarized in supplemental Table VI, we demonstrated by 3 different methods (ie, by feeding a cholesterol-rich diet, by RNA interference, and by deleting the endogenous gene) that a reduction of apoE expression in SMCs resulted in a downregulation of IL-6 and a corresponding rise in IGFBP expression, which was reversible by supplementing IL-6 to the culture medium. Thus, we provide clear evidence that chronic hypercholesterolemia has lasting metabolic effects on SMCs, which are not only

attributable to the inflammatory response in the vasculature but also influenced by the expression levels of apoE.

Metabolic Effects of Cytokines

Although metabolic disturbances are recognized as a key factor in both the initiation and progression of atherosclerosis, inflammatory cytokines are predominantly studied in the context of inflammation and their pronounced metabolic actions that contribute to the general adaptation of the organism during inflammatory stress are attracting less attention in cardiovascular research. IL-6 is known to play a key role in the immune and acute phase response. IL-6 expression is higher in atherosclerotic than normal arteries, and injection of IL-6 accelerates atherosclerosis in apoE$^{-/-}$ mice.[37] In addition to its potent inflammatory properties, however, IL-6 may have profound metabolic effects in vascular SMCs. In skeletal muscle, for example, IL-6 has been implicated as "exercise factor"[38,39] that acts in a paracrine manner on neighboring muscle cells and is released within minutes. The depletion of glycogen stores within muscle fibers triggers the release of IL-6 to increase glucose supply by stimulating lipolysis in the adipose tissue and glycogen breakdown in the liver.[39] In contrast to TNF-α, IL-6 does not make cells insensitive to insulin but stimulates glucose uptake and the rise of IL-6 during prolonged exercise can become as high as in sepsis.

Atherosclerotic lesions occur at preferential sites along the vasculature, and increased metabolic demand may explain why specific hemodynamic conditions initiate disease at these particular locations but not in their vicinity. We have demonstrated recently that mechanical stretch is a potent inducer of IL-6 in vascular SMCs.[24] We now report that IL-6 secretion is inversely correlated with glucose consumption: wild-type SMCs consumed less glucose but secreted more IL-6, whereas high glucose turnover in apoE$^{-/-}$ SMCs and progenitor-derived SMCs was associated with low levels of IL-6 secretion. Moreover, their hypoxia-induced increase in IL-6 secretion was attenuated by higher glucose concentrations in the culture medium, indicating that substrate availability constitutes an important factor determining cytokine release. By regulating IGFBP synthesis and modulating IGF availability, IL-6 may constitute an integral component of the inflammatory–metabolic interplay in vascular SMCs.

IL-18 is another important regulator in the homeostasis of energy intake.[40] IL-18$^{-/-}$ mice showed insulin resistance at the level of muscle and adipose tissue and increased fat deposition in the arterial walls.[40] Like IL-6,[37] IL-18 administration accelerated atherosclerosis in apoE$^{-/-}$ mice.[41] Moreover, a double knockout for IL-18 and apoE had reduced atherosclerosis despite higher cholesterol levels,[42] and overexpression of an IL-18 binding protein in apoE$^{-/-}$ mice prevented lesion development and promoted a stable plaque phenotype.[43] The increase in IL-18 transcription, as observed in the present study, is consistent with a recent report that apoE is a negative regulator of IL-18.[44] Overall, it is apparent that cytokines not only regulate immune or inflammatory responses in atherosclerosis but also contribute to glucose homeostasis[45] in the SMC compartment.

Therapeutic Potential of Resident Progenitor Cells

Our findings demonstrate that resident Sca-1^{+} progenitors from the aortic adventitia are a viable source of vascular SMCs and differentiate more readily toward the vascular SMC lineage than embryonic stem cell–derived progenitors. Remarkably, they shared many characteristics of apoE$^{-/-}$ SMCs, including their increased susceptibility to oxidative stress, although Sca-1^{+} cells were harvested from disease-free aortas of young apoE$^{-/-}$ mice and differentiated to SMCs in a normolipidemic environment. These data suggest that the deletion of the apoE gene has effects that extend beyond SMC differentiation to the progenitor level. In addition, besides reducing the number of circulating progenitors, vascular risk factors, such as hypercholesterolemia, may also influence the differentiation and regenerative potential of local stem cell populations, ie, by increasing hypoxia-regulated factors such as IGFBP-3,[35] a recently identified modulator of vascular survival and regrowth in an oxygen-deprived environment.[46]

Summary

By integrating multiple phenotypic facets of mature and progenitor cell-derived SMCs from hyperlipidemic mice, we illustrate how changes in the proteome, the secretome, and the metabolome are reciprocally connected and how proteomics offers an opportunity to progress toward a molecular classification of stem cell–derived cells.

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Disclosures

None.

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