SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

Human Subjects

The Diabetes Heart Study (DHS) is an affected sib-pair study of subclinical atherosclerosis and its risk factors, consisting of families with two or more siblings with a diagnosis of T2D and lacking advanced renal insufficiency. Additional diabetic or non-diabetic siblings were also recruited when available. Ascertainment and recruitment have been described previously[1, 2]. T2D was defined clinically as diabetes developing after the age of 35 years treated with insulin and/or oral agents, in the absence of historical evidence of ketoacidosis. The analyses here are restricted to 780 European-American sib-pairs with T2D from 375 families.

Protocols were approved by the Institutional Review Board of Wake Forest University School of Medicine, and all participants gave informed consent. Participant examinations were conducted in the General Clinical Research Center at Wake Forest University Baptist Medical Center, and included interviews for medical history, health behaviors, anthropometric measures, resting blood pressure, fasting total cholesterol, electrocardiography and spot urine collection. Common carotid artery intima-media thickness (IMT) was measured by high-resolution B-mode ultrasonography with a 7.5-MHz transducer and a Biosound Esaote (AU5) ultrasound machine as previously described[2]. Measurements were obtained using a well established protocol at Wake Forest University Baptist Medical Center, with correlations of repeat exams in the range of 0.88[3].

Isolation of Human Genomic DNA and Genotyping

Total genomic DNA was purified from whole blood samples obtained from subjects using the PUREGENE DNA isolation kit (Gentra, Inc.). DNA concentration was quantified using standardized fluorometric readings on a Hoefer DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech Inc.). Each sample was diluted to a final concentration of five ng/μl. Genotypes were determined using a MassARRAY SNP Genotyping System (Sequenom Inc.)[4]. This genotyping system uses single-base extension reactions to create allele-specific products that are separated and scored in a matrix-assisted laser desorption ionization/time of flight mass spectrometer. Primers for PCR reactions were designed using the MassARRAY Assay Design Software (Sequenom Inc.). Sequences are available by request.

Statistical Analysis of Human Genomic Data

Maximum likelihood allele and genotype frequencies for each tagSNP were calculated from unrelated probands and were tested for departures from Hardy-Weinberg equilibrium using both χ^2 and exact tests. Estimates of linkage disequilibrium between tagSNPs were determined by calculating pair-wise D' and r^2 statistics in unrelated individuals. In the DHS, approximately 400 microsatellite markers from a 10 cM genome-wide linkage scan were used to examine and correct self-reported familial relationships using PREST (Pedigree Relationship Statistical Test)[1, 5]. These 'corrected' sib-pair families were used for the current statistical genetic analyses.

All analyses were performed using variance components approaches as implemented in Merlin[6]. The primary analysis is the test of association between *each ID3* tagSNP and IMT. Each tagSNP was tested for association in a model that included age, sex, BMI, systolic blood pressure, LDL and HDL as covariates, incorporating a variance component into the model to account for correlation between relatives due to familial resemblance. After identifying the most strongly associated tagSNP (with $p<0.05$) independent of covariates, a second analysis was performed that included the original covariates and the associated SNP to re-evaluate the evidence for association at all other tagSNPs. Phenotypes included in these analyses were transformed to approximate conditional normality to reduce heterogeneity of residual phenotypic variance across SNP genotypes. Because of the strong evidence for a role of Id3 in atherosclerosis in our previous studies, these studies were considered hypothesis-based tests and therefore no further adjustments were made.

Analysis of Id3 Expression of Id3105A vs Id3105T

Plasmid constructs expressing products of the ancestral (Id3105A) and variant (Id3105T) alleles of *ID3* were generated as detailed in the expanded methods section of the online data supplement. NIH3T3 fibroblast cells (2.3×10^5 per dish) were plated in 60 mm dishes 24 hours prior to transfection. Cells were transiently transfected with 0.6 μg of the Id3 plasmid (either the Id3105A or Id3105T construct) or 0.6 μg of GFP plasmid using the Effectene Transfection Reagent (Qiagen). Forty-eight hours after transfection, cells were collected by trypsinization, lysed and immunoblotted as previously described[7] (see supplementary data). Membranes were probed with a rabbit monoclonal antibody against Id3 (CalBIO), then stripped and re-probed with a rabbit polyclonal antibody against ShBle (InVivoGEN). Experimental findings were repeated three times in triplicate. Densitometric analysis was performed and statistical significance was determined using an unpaired t-test.

Co-immunoprecipitation

E12, Id3105A, Id3105T and ITF2B constructs were generated as described in the expanded methods section of the online data supplement. NIH3T3 cells (6×10^5 per dish) were plated in 10 cm dishes 24 hours prior to transfection. Cells were transfected with 1.0 μg of FLAG-E12 and 0.3 μg of Id3105A or Id3105T using the Effectene Transfection Reagent (Qiagen). Forty-eight hours after transfection, cells were trypsinized, washed in PBS and harvested in 500 μl lysis buffer Twenty-five μl of Gamma Bind G Sepharose beads (GE Healthcare) were incubated for 60 minutes with 1 μg of a monoclonal Id3 antibody generated by our laboratory. Co-immunoprecipitation and immunoblotting were then carried out as described previously and in the online data supplement[7]. Membranes were probed with monoclonal antibodies against FLAG (Sigma) or Id3 (CalBIO). Experimental findings were repeated in triplicate. Quantitative densitometric analysis was performed and the ratio of binding was determined as previously described[8, 9]. Statistical significance was determined using an unpaired t-test.

Promoter-Reporter Studies

Smooth muscle alpha actin (SMaA) promoter reporter constructs have been described previously[10, 11]. NIH3T3 fibroblast cells (6×10^4 per well) were plated in 6-well plates 24 hours prior to transfection. Cells were transiently transfected with 0.15 μg of smooth muscle alpha actin promoter-chloramphenicol acetyltransferase reporter construct (pCAT-SMaA) along with 2.7 μg of expression vectors (pEF4 empty vector, pEF4-FLAG-E12, pEF4-Id3105A or pEF4-Id3105T) using the FUGENE HD transaction reagent (Roche). Thirty-six hours after transfection, cells were lysed and assayed for chloramphenicol acetyltransferase (CAT) activity using a CAT ELISA kit as per the manufacturer's instructions (Roche). Protein content of individual samples was quantitated using a BioRad D_c Protein Assay and CAT values were normalized to protein. Additional replicate wells were harvested in parallel for Western blotting, which was carried out as above.

Microarray assays

Primary mouse aortic VSMCs were harvested as previously described[12]. Sample preparation and analysis were performed by the UVA GeneChip/Microarray Bioinformatics Facility. RNA was extracted and treated with DNase I. cDNA synthesis, labeling and array hybridization were carried out according to Affymetrix instructions (Affymetrix, Santa Clara, CA). Fold changes and p-values for sample comparisons were derived from the Microarray Analysis Suite (MAS v5.0) data analysis tool, invoking the Mann-Whitney nonparametric method. After confirmation of data quality, data sets were imported into Ingenuity Pathway Analysis software (Ingenuity Systems, Mountain View, CA) and biological pathways were analyzed. Differentially expressed genes that had a p-value less than 0.05 and a fold-change greater than or equal to 2.0 were not labeled as "absent" in microarray analysis for both groups and were included in the data analysis. Significant association of microarray data with cellular pathways was assessed as previously described[13]. Genes in pathways known to be involved in atherogenesis

(adhesion, immune trafficking, apoptosis, proliferation, and movement) that were significantly associated with our uploaded microarray data were then further analyzed for fold-change and significance.

Animal Subjects

All animal experiments were approved by the Animal Care and Use Committee at the University of Virginia. *Id3*$^{+/+}$*ApoE*$^{-/-}$ mice were purchased from Jackson laboratories (stock #002052). *Id3*$^{-/-}$ mice, a generous gift from Dr. Yuan Zhuang at Duke University, were backcrossed to C57BL/6 mice and purity of the resulting line was confirmed to be >99% based on microsatellite analysis. These mice were then bred onto the *ApoE*$^{-/-}$ background to create the *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice used in these studies. All mice were housed in microisolator cages in a pathogen-free facility. All mice were given water *ad libitum* and either a standard chow diet (Harlan Teklad #7012) or Western diet (Harlan Teklad #TD88137).

Assessment of Atherosclerosis in Mice

Beginning at eight to ten weeks of age, *Id3*$^{+/+}$*ApoE*$^{-/-}$ and *Id3*$^{-/-}$*ApoE*$^{-/-}$ mice were fed a Western diet for 16 or 32 weeks. Control animals were continued on a standard chow diet and euthanized after 16 or 32 weeks. Animals were anesthetized by intraperitoneal injection of ketamine/xylazine and the vasculature was perfused by left ventricular puncture using 4% paraformaldehyde. Aortic arch segments were embedded in paraffin and 5 μm thick serial sections were generated from the aortic valve to the bifurcation of the brachiocephalic artery in each animal. Sections were stained at 150 μm intervals using the Movat method[14] and plaque volume was assessed using ImagePro 5.0 software. Descending aortas were opened longitudinally, pinned and stained using Sudan IV as previously described[15]. Aortas were then imaged and lesion area was quantified using ImagePro 5.0. For analysis of plaque composition, three 5 μm cross sections per animal were stained by the Movat method. Areas that were non-nucleated and lacked the green mucin stain were considered acellular. Nucleated and mucin positive areas were considered cellular. These areas were measured using ImagePro software. Statistical significance was determined using a Mann-Whitney U-test.

Measurement of Serum Parameters in Mice

Glucose measurements were obtained from whole blood by tail snip using an AccuCheck Advantage glucometer. Lipid values were assayed from sera acquired at the time of sacrifice. Insulin levels were determined by radioimmunoassay (catalog #SRI-13K, Millipore). Statistical significance was determined using the Mann-Whitney U test.

SUPPLEMENTARY FIGURES AND TABLES

Supplementary Table I. Patient Characteristics in the Diabetes Heart Study.

Variables	Overall (n=780)
Age (years)	61.80 ± 9.20
Men (46.5%)	
Age (years)	62.50 ± 9.0
Women (53.5%)	
Age (years)	61.30 ± 9.3
Post-menopausal (%)	89.70
Hormone replacement therapy (%)	26.10
BMI (kg/m^2)	32.40 ± 6.80
Systolic BP (mm Hg)	139.8 ± 19.0
Diastolic BP (mm Hg)	72.80 ± 10.2
Current smoker (%)	16.30
Past smoker (%)	42.70
Pack years	21.50 ± 29.2
Lipid lowering medication (%)	44.70
HDL (mg/dl)	42.70 ± 12.2
LDL (mg/dl)	104.1 ± 32.4
Triglycerides (mg/dl)	214.2 ± 145
HbA1c (%)	7.600 ± 1.80
Fasting glucose (mg/dl)	149.8 ± 57.0
IMT	0.680 ± 0.13
Measures restricted to persons with diabetes	
Duration of diabetes (years)	10.2 ± 6.9
Treatment for diabetes	
Insulin (%)	26.20
Oral hypoglycemic (%)	76.00

Values are the average ± standard deviation

Supplementary Table II. Genes upregulated (> 4-fold) in *Id3*$^{-/-}$ smooth muscle cells.

Affymetrix	Symbol	Entrez Gene Name	Fold Change	p-value
1418945_at	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)	28.8	0.043
450782_at	WNT4	wingless-type MMTV integration site family, member 4	24.9	0.046
460238_at	MSLN	mesothelin	22.2	0.022
416200_at	IL33	interleukin 33	12.1	0.011
421404_at	CXCL15	chemokine (C-X-C motif) ligand 15	11.0	0.018
418752_at	ALDH3A1	aldehyde dehydrogenase 3 family, memberA1	8.96	0.041
1449070_x_at	APCDD1	adenomatosis polyposis coli down-regulated 1	8.52	0.000
1426673_at	CDH3	cadherin 3, type 1, P-cadherin (placental)	8.10	0.007
1436448_a_at	PTGS1	prostaglandin-endoperoxide synthase 1	7.53	0.006
1449865_at	SEMA3A	sema domain, immunoglobulin domain, (semaphorin) 3A	7.47	0.012
1422833_at	FOXA2	forkhead box A2	7.35	0.031
1418304_at	PCDH21	protocadherin 21	7.20	0.030
1422779_at	SMPD3	sphingomyelin phosphodiesterase 3, neutral membrane	6.81	0.015
1434599_a_at	TJP2	tight junction protein 2 (zona occludens 2)	6.12	0.014
1416382_at	CTSC	cathepsin C	4.97	0.002
1452014_a_at	IGF1	insulin-like growth factor 1 (somatomedin C)	5.79	0.012
1448688_at	PODXL	podocalyxin-like	5.26	0.034
1419328_at	SEMA4F	sema domain, immunoglobulin domain, (semaphorin) 4F	4.88	0.031
1448656_at	CACNB3	calcium channel, voltage-dependent, beta 3 subunit	4.64	0.046
1417301_at	FZD6	frizzled homolog 6 (Drosophila)	4.57	0.040
1448831_at	ANGPT2	angiopoietin 2	4.30	0.021
1450850_at	EZR	ezrin	4.27	0.009
1422789_at	ALDH1A2	aldehyde dehydrogenase 1 family, member A2	4.20	0.028
1451335_at	PLAC8	placenta-specific 8	4.02	0.005

Supplementary Table III. Genes downregulated (> 4-fold) in *Id3*$^{-/-}$ smooth muscle cells.

Affymetrix	Symbol	Entrez Gene Name	Fold Change	p-value
1419100_at	SERPINA3	serpin peptidase inhibitor, clade A member 3	-24.5	0.046
1460258_at	LECT1	leukocyte cell derived chemotaxin 1	-14.9	0.032
1448742_at	SNAI1	snail homolog 1 (Drosophila)	-13.5	0.012
1424007_at	GDF10	growth differentiation factor 10	-13.2	0.001
1423294_at	MEST	mesoderm specific transcript homolog (mouse)	-13.2	0.003
1418666_at	PTX3	pentraxin-related gene, rapidly induced by IL-1β	-12.4	0.001
1432466_a_at	APOE	apolipoprotein E	-12.1	0.000
1427038_at	PENK	proenkephalin	-11.7	0.005
1419527_at	COMP	cartilage oligomeric matrix protein	-10.1	0.010
1418061_at	LTBP2	latent transforming growth factor beta binding protein 2	-9.50	0.042
1451537_at	CHI3L1	chitinase 3-like 1 (cartilage glycoprotein-39)	-8.49	0.003
1417917_at	CNN1	calponin 1, basic, smooth muscle	-7.56	0.000
1419608_a_at	MIA	melanoma inhibitory activity	-7.53	0.008
1416855_at	GAS1	growth arrest-specific 1	-7.43	0.003
1425582_a_at	EMCN	endomucin	-6.81	0.049
1419383_at	S100B	S100 calcium binding protein B	-6.67	0.040
1449863_a_at	DLX5	distal-less homeobox 5	-6.40	0.037
1421287_a_at	PECAM1	platelet/endothelial cell adhesion molecule	-6.28	0.026
1419309_at	PDPN	podoplanin	-6.09	0.009
1452072_at	MYCT1	myc target 1	-5.88	0.027
1449580_s_at	HLA-DMB	major histocompatibility complex, class II, DM beta	-5.74	0.016
1451538_at	SOX9	SRY (sex determining region Y)-box 9	-5.62	0.013
1417936_at	CCL9	chemokine (C-C motif) ligand 9	-5.46	0.028
1419589_at	CD93	CD93 molecule	-5.41	0.024
1433956_at	CDH5	cadherin 5, type 2 (vascular endothelium)	-5.39	0.022
1416287_at	RGS4	regulator of G-protein signaling 4	-5.38	0.001
1449106_at	GPX3	glutathione peroxidase 3 (plasma)	-5.24	0.006
1448950_at	IL1R1	interleukin 1 receptor, type I	-5.17	0.049
1450440_at	GFRA1	GDNF family receptor alpha 1	-5.09	0.031
1416321_s_at	PRELP	proline/arginine-rich end leucine-rich repeat protein	-4.97	0.010
1425457_a_at	GRB10	growth factor receptor-bound protein 10	-4.96	0.035
1424131_at	COL6A3	collagen, type VI, alpha 3	-4.95	0.040
1448169_at	KRT18	keratin 18	-4.94	0.016
1423428_at	ROR2	receptor tyrosine kinase-like orphan receptor 2	-4.87	0.018
1426032_at	NFATC2	nuclear factor of activated T-cells, cytoplasmic, 2	-4.75	0.038
1417359_at	MFAP2	microfibrillar-associated protein 2	-4.52	0.008
1416006_at	MDK	midkine (neurite growth-promoting factor 2)	-4.46	0.002
1450325_at	ANGPT4	angiopoietin 4	-4.44	0.026
1451331_at	PPP1R1B	protein phosphatase 1, regulatory (inhibitor) subunit 1B	-4.35	0.045
1448136_at	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2	-4.28	0.004
1450509_at	CHST11	carbohydrate (chondroitin 4) sulfotransferase 11	-4.11	0.018
1448862_at	ICAM2	intercellular adhesion molecule 2	-4.06	0.023
1420377_at	ST8SIA2	ST8 α-N-acetyl-neuraminide α-2,8-sialyltransferase 2	-4.04	0.018

Supplementary Table IV. Average random plasma lipid, glucose and insulin profiles of *Id3*$^{+/+}$ *ApoE*$^{-/-}$ and *Id3*$^{-/-}$ *ApoE*$^{-/-}$ mice.

	Id3$^{+/+}$ *ApoE*$^{-/-}$	*Id3*$^{-/-}$ *ApoE*$^{-/-}$	p-value
Chow Fed			
Weight (g)	31.89 ± 0.42	30.90 ± 0.77	n.s.
Total cholesterol (mg/dL)	535 ± 36	512 ± 46	n.s.
LDL (mg/dL)	481 ± 33	360 ± 45	n.s.
HDL (mg/dl)	31 ± 2	33± 3	n.s.
Triglycerides (mg/dL)	136 ± 15	121± 15	n.s.
Glucose (mg/dL)	129 ± 9	137 ± 9	n.s.
Insulin (ng/mL)	0.96 ± 0.17	1.00 ± 0.20	n.s.
Western Fed			
Weight (g)	35.76 ± 1.60	33.33 ± 1.83	n.s.
Total cholesterol (mg/dL)	1553 ± 184	1480 ± 188	n.s.
LDL (mg/dL)	1492 ± 176	1426 ± 190	n.s.
HDL (mg/dl)	23 ± 2	25 ± 4	n.s.
Triglycerides (mg/dL)	216 ± 43	174 ± 22	n.s.
Glucose (mg/dL)	159 ± 6	138 ± 5	$p < 0.008$
Insulin (ng/mL)	2.38 ± 0.49	3.20 ± 0.83	n.s.

Values are the average ± standard deviation
n.s.: not significant

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