METHODS

Animal Studies: Anti-sense oligonucleotides (ASO) Selection

ASOs were produced as previously described [1]with modifications. Briefly, rapid throughput screens with ~80 ASOs against mouse SCD-1 were performed and reduction of target gene expression was analyzed with real-time quantitative RT-PCR after transfection of the cells with different concentrations of the ASOs for 24h. Based on IC50 values, 3-4 potent ASOs were selected and their *in vivo* activity was confirmed in lean mice. The final selection of the SCD-1 ASO (ISIS 185222) was based on the maximal reduction of hepatic SCD-1 mRNA levels in lean mice. A negative control ASO (ISIS141923), which has the same chemical composition as ISIS185222 but does not exhibit perfect complementarity to any known gene sequence in public databases, was also included in the studies. Both ASOs were synthesized as 20-base phosphorothioate chimeric ASOs, where bases 1-5 and 16-20 had a 2'-O-(2-methoxyethyl) modification. This chimeric design provides increased nuclease resistance and mRNA affinity, while maintaining the RNAase H terminating mechanism, as described previously [1].

Animal Studies: Experimental Design

A total of 24 wild-type, 8 week-old male C57BL/6J mice purchased from Jackson Laboratory (Bar Harbor, Maine) were used in the study. The study was approved by the Johns Hopkins University Animal Care and Use Committee and complied with the American Physiological Society Guidelines for Animal Studies. For blood sample collection, surgical procedures, and tissue collection anesthesia was induced and

maintained with 1-2 % isoflurane administered through a facemask. Mice were fed a high cholesterol diet (TD88051, Teklad WI, 4 kcal/g, 15.8% fat, and 1.25% cholesterol).

A gas control delivery system was designed to regulate the flow of room air, nitrogen and oxygen into customized cages housing the mice as previously described [2]. During each period of IH, the FIO_2 was reduced from 20.9 to 4.9 $\pm$ 0.1 % over a 30 s period and then rapidly reoxygenated to room air levels in the subsequent 30 s period. The use of multiple inputs into the cage produced a uniform nadir FIO_2 level throughout the cage.

Twelve mice were placed in the IH chamber for ten consecutive weeks. Twelve mice on were placed in an identical chamber, but received intermittent air (IA) at the identical flow rate. During the last six weeks of exposure, six mice from the CIH group and six mice from the IA group were treated with SCD-1 ASO. Six mice from the CIH group and six mice from the IA group were treated with scrambled control ASO i.p. at a dose of 25/mg/kg twice a week. Animals were kept in a controlled environment (22-24 °C with a 12 h : 12 h light : dark cycle; lights on at 09.00) with free access to food and water. The IH and IA states were induced during the 12 h light phase alternating with 12 h of constant room air during the dark phase.

Mice fasted for 6 hrs prior to bleeding and sacrifice. Arterial blood (~1 ml) was obtained by direct cardiac puncture under 1-2 % isoflurane anesthesia. The heart and aorta were dissected. The atria with ascending aorta were separated and frozen in Sakura Tissue-Tek OCT Compound (Sakura Finetek USA, Inc. Torrance, CA). The descending aorta was

fixed in 10% paraformaldehyde. Livers were surgically removed and immediately frozen at -80^{o}C for future analysis.

Animal Studies: Histopathology

Serial 7-10 μm-thick sections were prepared from the proximal 1 mm of the aortic origin. Ten-fifteen sections were collected at 50 μm intervals starting from the appearance of the aortic valves, thaw-mounted on slides and air dried. After fixation in 4% paraformaldehyde (pH =7), the sections were stained with Meyer's hematoxylin and oil red-O (Sigma-Aldrich, USA) as previously described [3]. Images of the aorta were recorded using Olympus Camedia 5050 digital camera and stored as TIFF files of resolution 1024x768 pixels. Total area of the lesion was measured in each slide by using Image-Pro Discovery, version 5.1software from Media Cybernetics, Inc. (Silver Spring, MD) in a blinded fashion. ROS production in the aorta was assessed by the DHE stain [4]. Four frozen sections representing every eighth serial section were incubated with 10 μM of DHE (Molecular Probes Inc., Eugene, OR) for 5 min, rinsed, mounted, observed with an Olympus BX41 fluorescent microscope, using excitation at 535 nm an emission at 610, and quantified using NIH Image software.

Animal Studies: Enface Preparation and Oil Red-O staining of the aorta

The whole descending aorta fixed in 4% formaldehyde was separated from adventitia and surrounding adipose and connective tissues and processed as previously described [5] with minor modifications . Briefly, the dissected aorta was rinsed in 70% ethanol, immersed in a filtered solution containing 0-5% Sudan IV (Sigma Chemical Co),

35% ethanol, and 50% acetone, and destained in 80% ethanol. The Sudan-stained aortas were pinned out on a black wax surface, and the size of lesions in the aortas was quantified by using images of aortas captured with a Q-Color 3 color digital video camera (Olympus America, Canada) linked to the microscope. The captured images were analyzed and the lesion areas were measured with Image-Pro Discovery, version 5.1 software from Media Cybernetics, Inc. in a blinded fashion by one of the authors (VS).

Human Studies: Experimental Design

Nineteen consecutive patients (eighteen women and one man) were recruited from the Johns Hopkins Bayview Medical Center (JHBMC) Bariatric Surgery Clinic. The protocol was approved by the Western Institutional Review Board. All subjects gave informed consent. Patient inclusion criteria were age> 21yrs and a body mass index of > 35 kg/m^2. Exclusion criteria included: previous treatment of OSA within the prior 3 months, sleep disorders other than OSA (i.e., narcolepsy or periodic leg movements), previous upper airway surgery, recent weight loss of ≥10%, diabetes mellitus type 2 (defined by prior clinical diagnosis or use of hypoglycemic agents), therapy with any lipid lowering medications, current history of alcohol abuse, known chronic liver disease including non-alcoholic steatohepatitis, chronic viral hepatitis (B or C), iron overload (hemochromatosis), or cirrhosis of any etiology, history of HIV disease and/or current anti-HIV therapy, current systemic use of steroids, unstable cardiovascular disease (congestive heart failure, myocardial infarction or revascularization procedures, and unstable arrhythmias), uncontrolled hypertension (blood pressure > 190/110 mm Hg), significant lung disease with daytime hypoxemia or hypercapnea, supplemental oxygen

use, renal failure on dialysis, pregnancy, or bleeding disorders. One to three months prior to the bariatric surgery, patients were admitted to the Johns Hopkins Bayview General Clinical Research Center for a full-night sleep study. At the termination of the sleep study at 7 am, fasting blood samples were obtained for blood glucose, insulin levels and the lipid profile, and body anthropometry was performed. The AHI (number of episodes/hr) was computed as the number of apneas and hypopneas that occurred per hour of sleep. Prevalent OSA was defined by an AHI of >10 episodes/hr. OSA severity was defined by both the AHI and the average fall in oxyhemoglobin saturation (ΔSaO_2) during apneic/hypopneic episodes. A wedge liver biopsy was obtained by the surgeon the gastric bypass procedure and immediately frozen at -80^{o}C for future analysis.

Biochemical Analyses of Mouse and Human Samples

Plasma total cholesterol, triglycerides, and FFA were measured with kits from Wako Diagnostics, Inc. (Richmond, VA). Lipids were extracted from the mouse liver with chloroform- methanol, according to Bligh-Dyer procedure and measured using kits from Wako Diagnostics. Mouse fasting blood glucose was measured with Accu-Chek® Comfort Curve TM kit from Roche Diagnostics, Inc. (Indianapolis, IN). Plasma insulin levels in mice were detected with ELISA kits from Linco Research, Inc. (St. Charles, MO). Plasma lipoproteins from mice and human subjects were subjected to gel filtration HPLC on two tandemly connected TSK-Gel Lipopropak XL columns (300 x 7.8 mm) with simultaneous measurement of TG and cholesterol using an on-line dual detection system, according to *LipoSEARCH* technology (Skylight Biotech, Inc., Tokyo, Japan).

Plasma total fatty acids were analyzed as previously described [6] with minor modifications. Briefly, fatty acids were hydrolyzed, extracted with hexane, and derivatized with pentafluorobenzyl bromide. The resulting esters were resolved on capillary gas chromatography electron capture negative ion mass spectrometry (GC/MS). The analysis was performed on an Agilent 6890/5973 GC/MS operating in the negative ion mode using an SP-2560 capillary column (Supelco, Inc., Bellefonte, PA) and ammonia as the reagent gas. Each fatty acid was matched to the labeled internal standard of closest chain length, retention time, and concentration.

Real Time PCR in Mouse and Human Liver Tissue

Total RNA was extracted from liver using Trizol (Life Technologies, Rockville, MD) and cDNA was synthesized using Advantage RT for PCR kit from Clontech (Palo Alto, CA). Real-time reverse-transcriptase PCR (RT-PCR) was performed with primers from Invitrogen (Carlsbad, CA) and Taqman probes from Applied Biosystems (Foster City, CA). The sequences of primers and probes for mouse SCD-1 and 18S were previously described [7-9]. Efficiency of mouse SCD-1 real time PCR was 94.7 ± 3.4%, and efficiency of mouse 18S real time PCR was 99.9 ± 4.1%. The sequences of primers and probes for human SCD were designed based on the GeneBank sequence NM_005063, forward primer 5'- GCCCGCCAGTCA -3', reverse primer 5'- TGACCGTGTCCGGTATTTCC-3', and the probe 5'- TCGCACTTTGCCCC-3'. The sequences of primers and probes for human 18S were designed based the GeneBank sequence X03205, forward primer 5'- GCGGCTTTGGTGACTCTAGATAA -3', reverse primer 5'- ACGGCGACTACCATCGAAAGT -3', and the probe 5'-

ATTCGAACGTCTGCCC -3'. Efficiency of human SCD-1 real time PCR was 98.6 ± 4.7%, and efficiency of human 18S real time PCR was 101.0 ± 5.9%. The mRNA expression levels were normalized to 18S rRNA concentrations using the following formula: ΔCt = 18SCt – SCD Ct. Given that SCD/18S = $(2 \times E_{18S}/ E_{SCD})^{Ct(18S)-Ct(Gene\ of\ Interest)}$, where E_{SCD} and E_{18S} are SCD and 18S PCR efficiencies respectively, ΔΔCt of 1 in SCD-1/18S between two groups of mice corresponded to a 1.92 fold difference, and ΔΔCt of 1 in SCD/18S between two groups of patients corresponded to a 1.96 fold difference.

Immunoblot in Mouse and Human Liver Tissue

An aliquot of the liver tissue from each mouse was homogenized and the microsomal fraction was isolated as previously described [7]. Aliquots (70μg of protein) were analyzed by 4-15% SDS-PAGE followed by immunoblot assays using goat polyclonal antibodies against mouse SCD-1 (sc-14719) and rabbit polyclonal antibodies against mouse actin (sc-10731) from Santa Cruz Biotechnology (Santa Cruz, CA), Human livers were processed in an identical fashion. Immunoblot was performed using mouse monoclonal antibodies against human SCD (sc-58420) and rabbit polyclonal antibodies against human actin (sc-10731). Goat anti-mouse-HRP conjugate and goat anti-rabbit conjugate from BioRad (Hercules, CA) or bovine anti-goat-HRP conjugate from Santa Cruz were used as needed. Immunoblot was developed using ECL system from Amersham (Piscataway, NJ). Densitometry was performed using ChemiDoc XRS system from BioRad (Hercules, CA) and UN-SCAN-IT Gel Automated Digitizing System, version 5.1 software (Silk Scientific Corporation, Orem, UT). The results were expressed as ratios of

optical density of the bands representing SCD to optical density of the band representing actin.

Statistical Analyses

All values are reported as mean ± SEM. Statistical comparisons between four groups of mice (CIH-control ASO, IA-control ASO, CIH-SCD-1 ASO, IA-SCD-1 ASO) were performed by a general linear model ANOVA across two independent variables, hypoxia and ASO, followed by the Tukey's post-hoc test. Comparisons between Day 0, Day 30 and Day 84 of mouse exposure were performed using repeated-measures ANOVA. Human subjects were divided in two groups according to a median BMI, AHI, or Δ SaO_2 and analyzed using an unpaired *t*-test. A p-value of less than 0.05 was considered significant. Linear regression analysis was used to assess relationships between Δ SaO_2 and SCD mRNA.

All authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Reference List

(1) McKay RA, Miraglia LJ, Cummins LL, Owens SR, Sasmor H, Dean NM. Characterization of a potent and specific class of antisense oligonucleotide

inhibitor of human protein kinase C-alpha expression. *J Biol Chem.* 1999; 274:1715-22.

(2) Polotsky VY, Li J, Punjabi NM, Rubin AE, Smith PL, Schwartz AR, O'Donnell CP. Intermittent hypoxia increases insulin resistance in genetically obese mice. *J Physiol.* 2003; 552:253-64.

(3) Zhang SH, Reddick RL, Burkey B, Maeda N. Diet-induced atherosclerosis in mice heterozygous and homozygous for apolipoprotein E gene disruption. *J Clin Invest.* 1994; 94:937-45.

(4) Barry-Lane PA, Patterson C, van der MM, Hu Z, Holland SM, Yeh ET, Runge MS. p47phox is required for atherosclerotic lesion progression in ApoE(-/-) mice. *J Clin Invest.* 2001; 108:1513-22.

(5) Tangirala RK, Rubin EM, Palinski W. Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice. *J Lipid Res.* 1995; 36:2320-8.

(6) Lagerstedt SA, Hinrichs DR, Batt SM, Magera MJ, Rinaldo P, McConnell JP. Quantitative determination of plasma c8-c26 total fatty acids for the biochemical diagnosis of nutritional and metabolic disorders. *Mol Genet Metab.* 2001; 73:38-45.

(7) Li J, Thorne LN, Punjabi NM, Sun CK, Schwartz AR, Smith PL, Marino RL, Rodriguez A, Hubbard WC, O'Donnell CP, Polotsky VY. Intermittent hypoxia induces hyperlipidemia in lean mice. *Circ Res.* 2005; 97:698-706.

(8) Li J, Grigoryev DN, Ye SQ, Thorne L, Schwartz AR, Smith PL, O'Donnell CP, Polotsky VY. Chronic intermittent hypoxia upregulates genes of lipid biosynthesis in obese mice. *J Appl Physiol.* 2005; 99:1643-8.

(9) Li J, Nanayakkara A, Jun J, Savransky V, Polotsky VY. The Effect of Deficiency in SREBP Cleavage-Activating Protein (SCAP) on Lipid Metabolism during Intermittent Hypoxia. *Physiol Genomics.* 2007; 31:273-80.

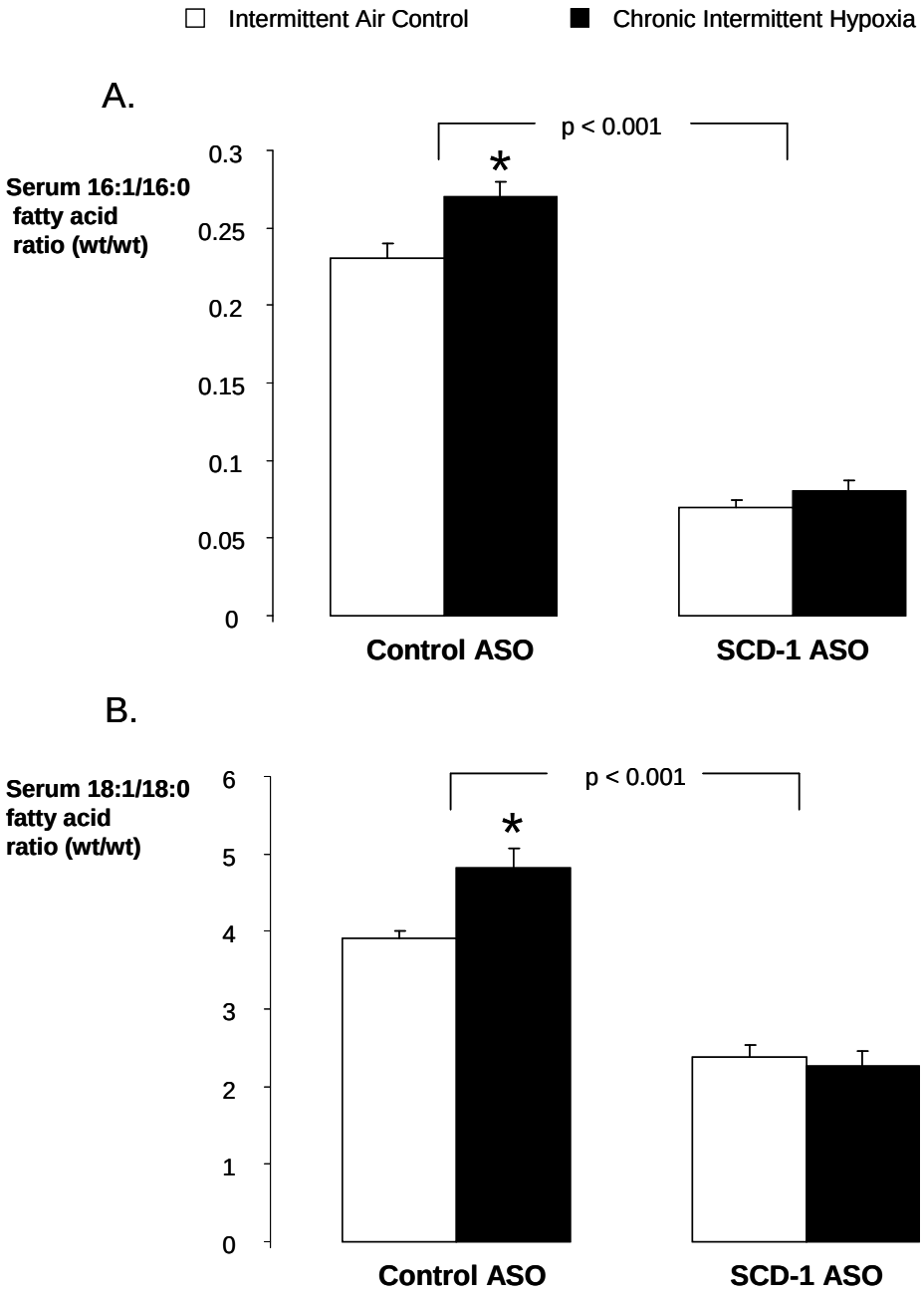
Online Table I. Basic, sleep, and metabolic characteristics of obese patients undergoing bariatric surgery

	Body mass index (kg/m^2)		Apnea-hypopnea index (events/hr)		ΔSaO_2 (%)	
	< 48.7	≥48.7	≤ 14	> 14	< 5.0	≥ 5.0
Age	42.8±2.4	39.6±2.5	38.3±2.3	46.2±2.0*	38.2±2.7	44.7±1.4*
BMI (kg/m_2)	44.7±1.1	51.8±1.5‡	48.1±1.4	49.6±2.6	45.9±1.3	50.5±1.9
Neck (cm)	39.7±0.8	44.0±1.0†	41.2±1.1	42.4±1.1	39.6±0.6	44.1±1.0†
Waist (cm)	132±10.8	140±3.3	140±9.5	131±5.5	133±10.6	137±3.8
AHI (events/hr)	24.6±8.5	27.5±10.4	6.8±1.3	49.4±8.5‡	14.0±6.3	39.2±10.4
Baseline SaO_2 (%)	95.8±0.7	95.6±0.4	96.3±0.3	94.9±0.6	96.4±0.3	94.9±0.6
Average low SaO_2 (%)	90.9±1.0	90.1±1.0	91.9±0.6	88.2±1.2*	92.5±0.5	88.3±1.0†
ΔSaO_2 (%)	4.9±0.5	5.5±0.6	4.4±0.4	6.7±0.7*	3.9±0.3	6.6±0.4‡
SCD mRNA (ΔCt)	-14.2± 0.5	-11.9± 1.0	-13.4± 0.7	-12.8± 0.9	-14.8± 0.4	-11.3± 0.7‡
Fasting plasma cholesterol (mg/dl)	195 ± 2.8	185 ± 10	186 ± 15	193 ± 6	175 ± 10	204 ± 8*
Fasting plasma triglycerides (mg/dl)	86.8± 10	111 ± 10	94.6± 22	101 ± 14	66.6 ± 4.5	125 ± 18*
Fasting free fatty acids (mmol/l)	0.63±0.07	0.44±0.04	0.44±0.08	0.50±0.02	0.54±0.06	0.51±0.06

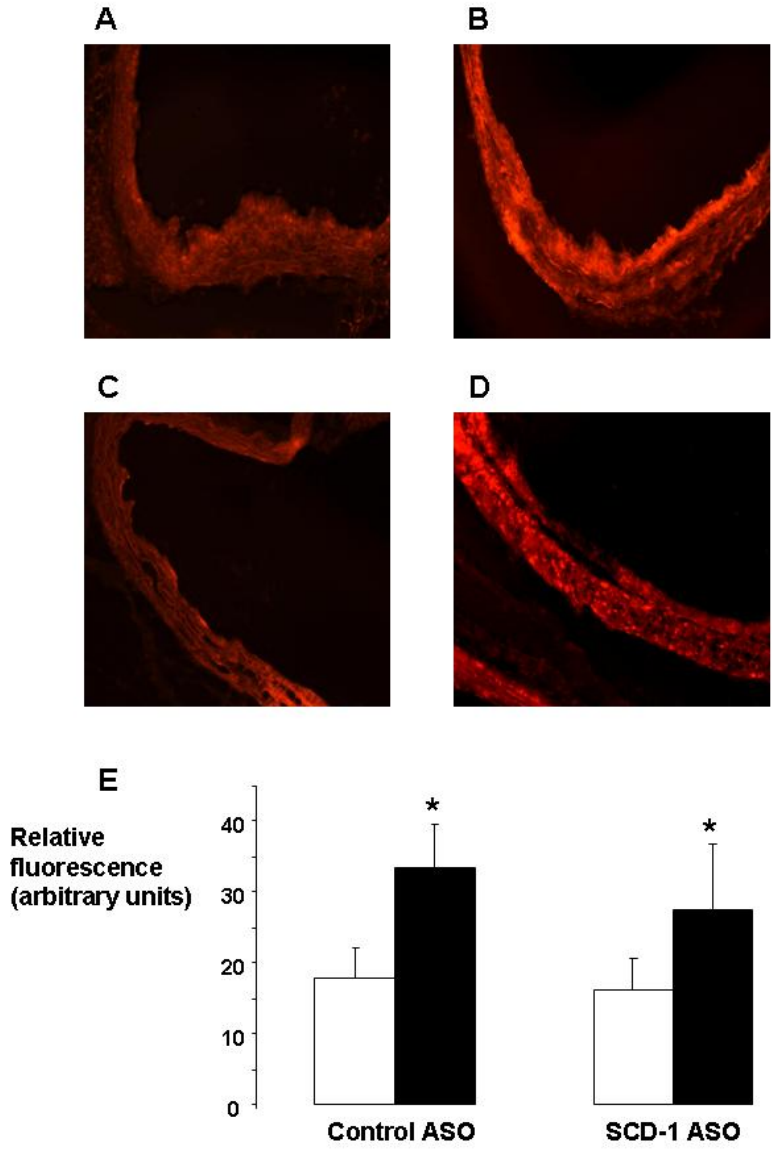
SaO_2, oxyhemoglobin saturation; ΔSaO_2 – difference between baseline SaO_2 and average low SaO_2 during apnic events ; SCD, hepatic stearoyl coenzyme A desaturase; Ct, the critical threshold cycle; Δ Ct is the difference between 18S and SCD Ct values; *, †, and ‡ denote $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, between two groups of patients.

Online Figure I. Fatty acid molecular species were identified via combined Gas chromatography–mass spectrometry (GC-MS) analysis. GC-MS was performed in plasma of C57BL/6J mice receiving SCD-1 or control ASO and exposed to CIH or IA for 10 weeks. The 16:1 (n-9)/16:0 *(A)* and the 18:1 (n-9)/18:0 *(B)* ratios of fatty acids were calculated for each condition. * denotes $p < 0.05$ for the difference between CIH and IA.

Online Figure II. Representative cross-sections of the ascending aorta (sinus of Valsalva) in C57BL/6J mice exposed to (*A*) intermittent air (IA) and control anti-sense oligonucleotides (ASO) injections, (*B*) chronic intermittent hypoxia (CIH) and control ASO, (*C*) IA and SCD-1 ASO, or (*D*) CIH and SCD-1 ASO. Transverse frozen sections of the aorta were stained with dihydroethidium (DHE) for 10 min. Original magnification: x100. (*E*) shows mean relative fluorescence of the aortic sections; *solid bars* = CIH; *open bars* = IA. * denotes $p < 0.05$ for the difference between CIH and IA.



Savransky et al. Online Figure I



Savransky et al. Online Figure II