SUPPLEMENTARY INFORMATION

SUPPLEMENTARY TABLES

Table S1: Primer sequences for porcine genes and PCR conditions.

Gene	Primer Sequence	PCR Annealing Temp.(°C)	PCR Extension Time (s)	Mg^{2+} Conc. (mM)
GAPDH	F: 5'GGG CGA TGC TGG TGC TGA GTA AGT3' R: 5'ACG TTG GCA GTA GGG ACA CGG AAG3'	63	18	4
IRE1α	F: 5' CAA GAG CAA GCT CAC G3' R: 5' AGA AGC CAG TAG TTC CT3'	59	10	3
PERK (EIF2AK3)	F: 5' CCA GCC TTA GCA AAC C3' R: 5' ACT ATA TGC ACT GAG TCC G3'	59	10	3
ATF6 α	F: 5' CAT CAG AGC CGC TAA AG3' R: 5' ACG TGA TTA GGT AGC TGT3'	58	15	4
HSPA5 (GRP78/BiP)	F: 5' CCT ACT CGT GCG TTG G3' R: 5' CTG CAC AGA CGG GTC A3'	59	10	4
CHOP	F: 5' TAT TTA TTA CTG CCC AAA TCC AT3' R: 5' CAA CAT TGT CCG AGA ATT GA3'	59	10	4
ATF4	F: 5'TAA CCG ACA AAG ACA CCT 3' R: 5'GGC ATC GAA GTC GAA C3'	59	14	5
XBP1	F: 5'TGT AGA CCA TTC GTG GG 3' R: 5'GGG TGA CCT ATG AGG TT 3'	59	10	4
NRF2	F: 5'CCA AGA CCA CCG TGA A 3' R: 5'GGT GAC AGG GGT TGG A 3'	59	12	5
VCAM1	F:5'CAGCCCTCAGTAAAGACAACACCA 3' R:5'GTCATCATCACGGAGTCACCTTCT 3'	65	10	4

Table S2: Gene Set Enrichment Analysis of differential endothelial gene expression in athero-susceptible regions: Enriched Gene Ontology Cellular Component (A), Gene Ontology Molecular Function (B) and Gene Ontology Biological Process (C) gene sets with less than 30% FDR in in athero-susceptible endothelium compared to athero-protected endothelium are shown.

GO Term	Gene Sets	FDR
A)		
GO Cellular Component gene sets		
GO:0044432	endoplasmic reticulum part	0.036
GO:0005789	endoplasmic reticulum membrane	0.092
GO:0042175	nuclear envelope-endoplasmic reticulum network	0.101
GO:0031227	intrinsic to endoplasmic reticulum membrane	0.126
GO:0005783	endoplasmic reticulum	0.138
GO:0000502	proteasome complex (sensu Eukaryota)	0.163
GO:0030176	integral to endoplasmic reticulum membrane	0.166
GO:0048471	perinuclear region of cytoplasm	0.190
GO:0008287	protein serine/threonine phosphatase complex	0.232
GO:0031301	integral to organelle membrane	0.243
GO:0031300	intrinsic to organelle membrane	0.259
B)		
GO Molecular Function gene sets		
GO:0008639	small protein conjugating enzyme activity	0.183
GO:0051082	unfolded protein binding	0.275
C)		
GO Biological Process gene sets		
GO:0000245	spliceosome assembly	0.233
GO:0006986	response to unfolded protein	0.245
GO:0046034	ATP metabolic process	0.255
GO:0051789	response to protein stimulus	0.275

Table S3: Subset of upregulated genes in athero-susceptible regions of arteries. FDR refers to false discovery rate as determined by differential gene expression analysis (PaGE) for each gene. Expression ratio of each endothelial gene is the unlogged difference of normalized M values for athero-susceptible to athero-protected regions.

Gene Symbol/TC annotation		Gene identification	FDR	Expression Ratio
mRNA Processing				
spliceosome complex				
HNRNPAB	TC58919	Heterogeneous nuclear ribonucleoprotein A/B	0.165	1.20
HNRPDL	TC47918	Heterogeneous nuclear ribonucleoprotein D-like	0.164	1.23
HNRPM	TC58283	Heterogeneous nuclear ribonucleoprotein M	0.104	1.21
HNRPU	TC48046	Heterogeneous nuclear ribonucleoprotein U	0.165	1.16
PTBP1	TC57012	Polyprimidine tract binding protein 1	0.127	1.16
SFPQ	TC51161	Splicing factor proline/glutamine-rich	0.017	1.35
SYNCRIP	TC63028	Synaptotagmin binding, cytoplasmic RNA interacting protein	0.060	1.21
translation initiation and elongation				
DDX3X	TC54310	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	0.142	1.47
DHPS	TC49450	Deoxyhypusine synthase	0.218	1.14
EEF1E1	TC48981	Eukaryotic translation elongation factor 1 epsilon 1	0.060	1.23
EIF3S	TC48255	Eukaryotic translation initiation factor 3, subunit 2	0.101	1.23
Protein folding and glycosylation				
Folding				
PDIA4	TC47721	Protein disulfide isomerase family A, member 4	0.022	1.33
PDIA5	TC52004	Protein disulfide isomerase family A, member 5	0.022	1.25
PPID	TC63911	Peptidylprolyl isomerase D	0.099	1.17
molecular chaperones				
DNAJB6	TC59176	DNAJ (Hsp40) homolog, subfamily B, member 6	0.126	1.17
DNAJB9	TC60398	DNAJ (Hsp40) homolog, subfamily B, member 9	0.131	1.23
DNAJC13	TC63581	DNAJ (Hsp40) homolog, subfamily C, member 13	0.165	1.17
HSPA4	TC54801	Heat shock 70kDa protein 4 isoform	0.059	1.30
HSPA5	TC49389	Heat shock 70kDa protein 5	0.102	1.76
HSPH1	TC61428	Heat shock 105kDa/110kDa protein 1	0.104	1.86
misfolded protein binding in the cytosol				
CCT4	TC47241	Chaperonin containing TCP1 subunit 4	0.104	1.16
CCT6A	TC59175	Chaperonin containing TCP1, subunit 6A	0.066	1.23
CCT8	TC58096	Chaperonin containing TCP1, subunit 8	0.066	1.27
misfolded protein binding in the ER				
CALR	TC50246	Calreticulin	0.165	1.29
CANX	TC48027	Calnexin	0.188	1.17
CALU	TC49702	Calumenin	0.154	1.19

Protein transport/Endoplasmic Reticulum/Golgi Apparatus				
CNIH	TC58419	Cornichon homolog	0.078	1.22
KPNA2	TC59247	Karyopherin alpha 2	0.113	1.15
NUCB2	TC59174	Nucleobindin 2	0.245	1.13
SAR1B	TC62186	SAR1 gene homolog B	0.165	1.17
SEC61A1	TC60192	Sec61 alpha 1 subunit	0.092	1.27
SEC61B	TC51334	Sec61 beta subunit	0.245	1.19
SERP1	TC49953	Stress-associated endoplasmic reticulum protein 1	0.059	1.24
SURF4	TC49882	Surfeit 4	0.022	1.33
TPST1	TC50576	Tyrosylprotein sulfotransferase 1	0.189	1.17
Protein Degradation				
ANKRD45	TC53910	Ankyrin repeat domain 45	0.154	1.22
ASB8	TC57458	Ankyrin repeat and SOCS box-containing 8	0.066	1.34
HUWE1	TC59735	HECT, UBA and WWE domain containing protein 1	0.059	1.41
POMP	TC58556	Proteasome maturation protein	0.071	1.28
PSMD12	TC50442	Proteasome 26S subunit, non-ATPase, 12	0.245	1.13
RPN1	TC57602	Ribophorin I	0.127	1.20
SPPL2A	TC63526	Signal peptide peptidase-like 2A	0.108	1.15
UFD1L	TC56586	Ubiquitin fusion degradation 1 like	0.245	1.13
Lipid Synthesis				
ACSL4	TC60087	Acyl-CoA synthetase long-chain family member 4	0.193	1.19
PPAP2A	TC55996	Phosphatidic acid phosphatase type 2A	0.132	1.43
SC4MOL	TC59399	Sterol-C4-methyl oxidase-like	0.066	1.32
SQLE	TC60761	Squalene epoxidase	0.023	1.32

Table S4: Upregulated inflammatory and apoptosis genes in athero-susceptible regions of arteries. FDR refers to false discovery rate as determined by differential gene expression analysis (PaGE) for each gene. Expression ratio of each endothelial gene is the unlogged difference of normalized M values for athero-susceptible to athero-protected regions.

Gene Symbol/TC annotation		Gene identification	FDR	Expression Ratio
Anti-apoptosis				
ARMET	TC48748	Arginine-rich, mutated in early stage tumors	0.043	1.35
API5	TC58867	Apoptosis inhibitor 5	0.101	1.16
BIRC2	TC168828	Baculoviral IAP repeat-containing 2	0.071	1.30
NFIL3	TC51330	Nuclear factor, interleukin 3 regulated	0.244	1.21
Pro-inflammation				
GJA1	TC48199	Gap junction protein, alpha 1	0.010	1.83
VCAM1	TC57583	Vascular cell adhesion molecule 1	0.059	1.22
PTX3	TC63245	Pentraxin-related gene, rapidly induced by IL-1 beta	0.104	1.28
PLAT	TC61001	T-plasminogen activator	0.105	1.43
LITAF	TC59205	Lipopolysaccharide-induced TNF factor	0.072	1.24
THBS1	TC56674	Thrombospondin 1	0.188	1.43
Anti-inflammation				
ADM	TC49272	Pro-adrenomedullin	0.030	1.51
ID1	TC48038	Inhibitor of DNA binding/differentiation 1D	0.066	1.34
SERPINB6	TC63460	Serpin peptidase inhibitor, clade B (ovalbumin), member 6	0.066	1.28
TFPI2	TC59311	Tissue factor pathway inhibitor 2	0.165	1.41
SERPINI1	TC60274	Serpin peptidase inhibitor, clade I, member 1	0.169	1.28
LTBP1	TC51680	Latent transforming growth factor beta binding protein 1	0.189	1.27

SUPPLEMENTARY FIGURES

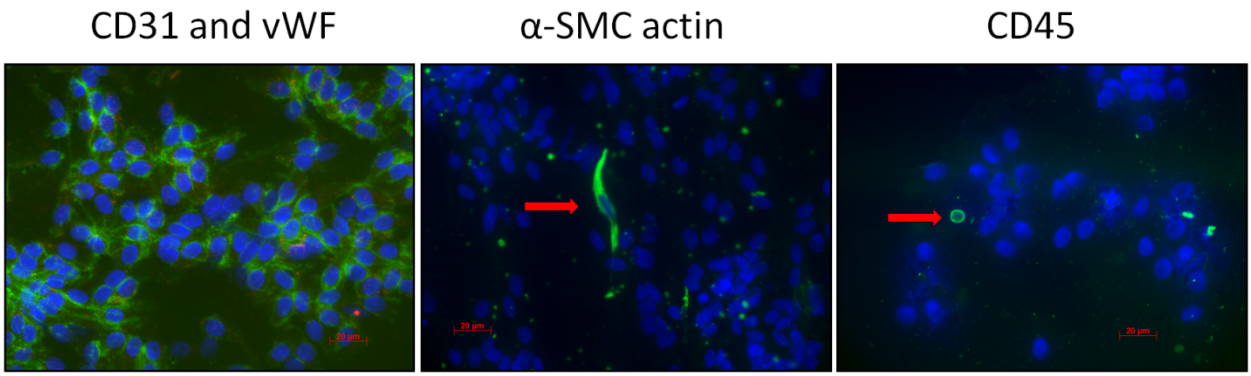


Figure S1. Purity of isolated endothelial samples. Scraped samples were fixed onto microscope slides. Purity and contamination was assessed by antibody staining. Left panel: CD31 (green) and von Willebrand Factor (vWF) (red) double staining for endothelial cells. Middle panel: α-smooth muscle actin (green) staining for smooth muscle. Right panel: CD45 (green) staining for leukocytes. Average endothelial purity was 96.5% with 2.78% smooth muscle cell and 0.72% leukocyte contamination. Nuclei were observed with blue Hoechst 33258 staining. Red arrows show a smooth muscle cell and a leukocyte. Red bars are 20 μm.

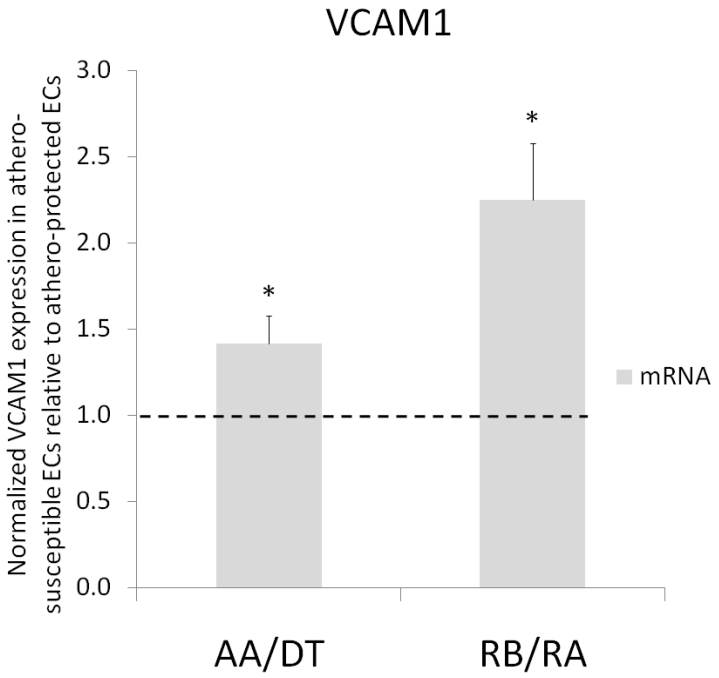


Figure S2. VCAM1 expression in athero-susceptible ECs compared to athero-protected ECs. VCAM1 gene expression was normalized to GAPDH. Ratio of expression in aortic arch (AA) to descending thoracic aorta (DT) and renal branch (RB) to renal artery (RA) was calculated for each paired sample based on their animal origin (n = 5 animals). Values > 1.0 indicate higher expression in athero-susceptible endothelium. Data represent mean±SEM. *$p \leq 0.05$ one-sample, one sided, paired Wilcoxon test.

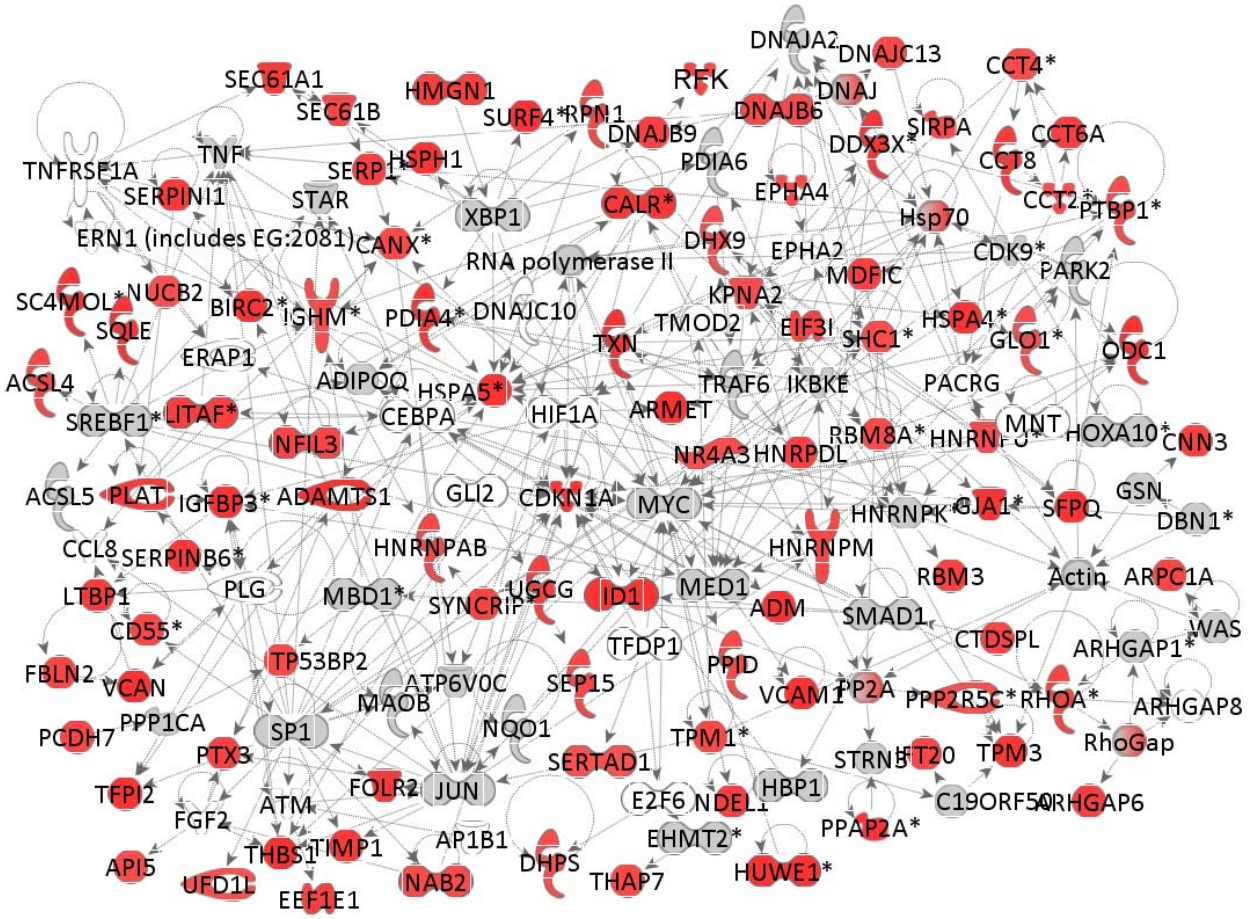
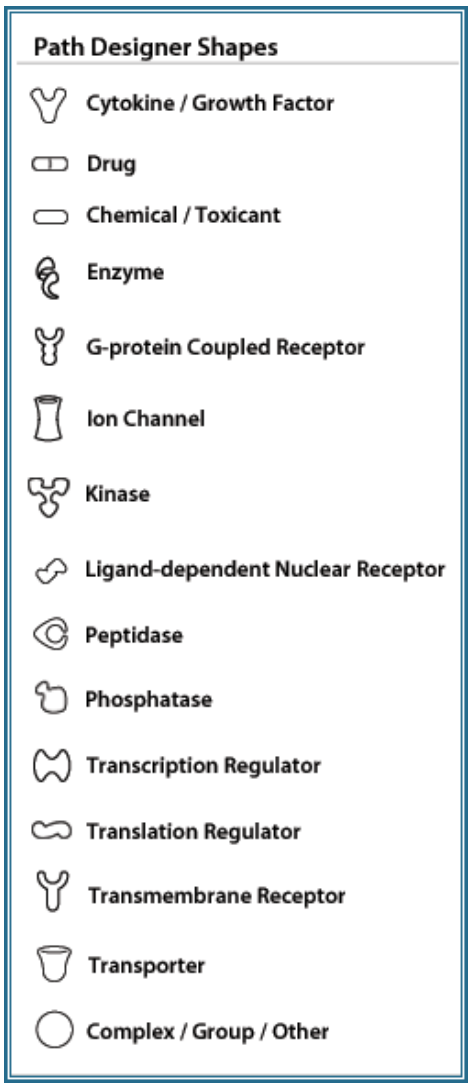
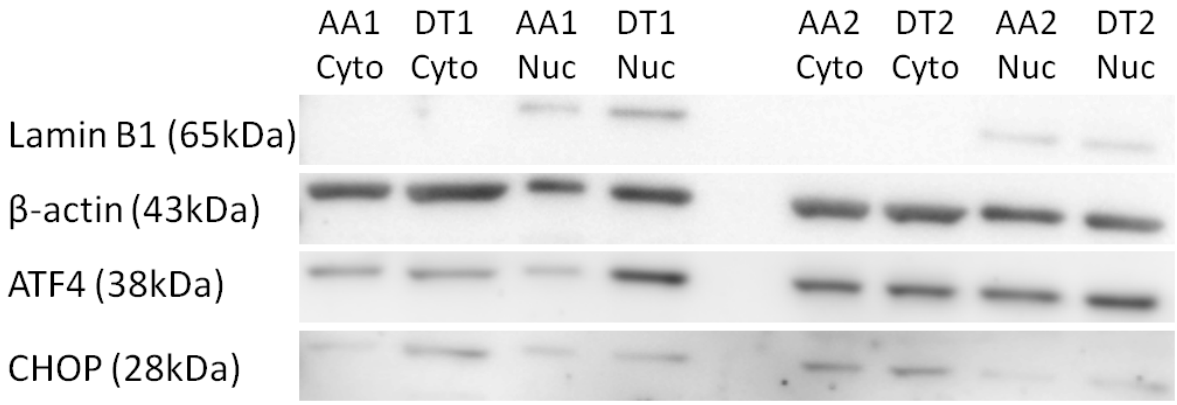


Figure S3: Ingenuity Pathway Analysis of endothelial gene expression. A dataset containing gene identifiers with corresponding expression values and FDRs were analyzed using Ingenuity Pathway Analysis. An FDR value cutoff of 25% was set to identify genes whose expression was significantly differentially regulated. Networks of these focus genes were then algorithmically generated based on their connectivity. Only direct interactions of gene-protein and protein-protein were considered. Seven networks were combined to form one large network. Red color indicates EC gene upregulation in athero-susceptible regions of arteries compared to athero-protected regions. Intensity of red is proportional to expression ratio. Gray color indicates molecules present in the data set that were not significantly differentially expressed (FDR > 25%)[1].



Legend for Supplementary Figure 2. Molecule shapes that identify each molecule in Supplementary Figure 2 are shown.

A.



B.

	AA1 Cyto	DT1 Cyto	AA1 Nuc	DT1 Nuc	AA2 Cyto	DT2 Cyto	AA2 Nuc	DT2 Nuc
ATF4/β-actin	4.4	4.6			2.2	3.0		
ATF4/Lamin B1			3.8	5.6			10.6	11.7
CHOP/β-actin	0.8	0.7			0.6	0.5		
CHOP/Lamin B1			1.8	2.1			2.7	6.7

Figure S4. ATF4 and CHOP expression in cytoplasmic (Cyto) and nuclear (Nuc) extracts. Endothelial isolates from 10 animals were pooled separately for aortic arch (AA) and descending thoracic aorta (DT). Protein Lamin B1 expression in 2 series (AA1, AA2 , DT1, DT2). (A) Western blot for the nuclear and cytoplasmic extracts of pooled endothelial cells. (B) Densitometric quantification of ATF4 and CHOP expression relative to β-actin and Lamin B1 for cytoplasmic and nuclear extracts, respectively.

SUPPLEMENTARY METHODS

1. Arterial Tissue Preparation

Ascending, descending and abdominal aortas with their branches, and carotid artery were harvested within 30 to 45 minutes after animal death. Vessel lumen was rinsed with ice-cold RNAase free PBS. Surrounding tissue was dissected and vessels were cut open longitudinally with artery scissors to prevent damage to endothelial cells. They were pinned onto waxed trays and rinsed once again with RNase-free PBS.

2. Endothelial cell harvest

Endothelial cells were gently scraped from discrete regions that ranged between 0.5 cm^2 and 1 cm^2 (5,000 to 10,000 cells). Sample collection was done at random over several days to prevent any sample collection and processing bias as well as other confounding factors. No more than three samples came from the same animal, in most cases each sample was from a single animal (55 animals in total). Scrapes from various regions were pooled to obtain a reference sample for microarray hybridizations.

3. Endothelial RNA extraction and quality control

Freshly isolated cells were transferred directly to a lysis buffer containing the RNase inhibitors guanidine isothiocyanate and β -mercaptoethanol (0.143 M) (Absolutely RNA Nanoprep Kit, Stratagene, La Jolla, CA) and stored on dry ice. Total RNA was isolated using the Absolutely RNA Microprep kit according to manufacturer's instructions. Briefly, equal volume of 70% RNAase-free ethanol was added to thawed cell lysates. They were loaded onto a silica-based fiber matrix, which binds RNA during centrifugation. Contaminating DNA was digested by a 15-min DNase treatment at 37 ^{0}C. Proteins and DNA were removed by high- and low-salt buffer washes. Total RNA was purified by two elutions of 50 µl in 65 ^{0}C elution buffer and RNAase-free water, successively.

Total RNA integrity was evaluated using an Agilent Bioanalyzer 2100 and RNA 6000 Nano Labchips (Agilent Technologies, Palo Alto, CA) according to manufacturer's instructions. Total RNA was judged to be intact if two ribosomal bands (28S and 18S) were present in approximately 2:1 ratio. RNA quantity was measured using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Inc., Rockland, DE). Total RNA with 260/280 ratio between 1.8 and 2.1 and concentrations higher than 10 ng/µl was used in subsequent procedures.

4. Messenger RNA amplification and evaluation

100 ng total RNA was amplified using the MessageAmp aRNA Kit (Ambion, Austin, TX). This kit is based upon the antisense RNA (aRNA) linear amplification procedure first described by Van Gelder and colleagues[2]. Poly(A) RNA was reverse transcribed with an oligo(dT) primer containing a T7 RNA polymerase promoter sequence. RNase H treatment cleaved the mRNA into small fragments that served as primers during second-strand synthesis, resulting in double-stranded cDNA template for T7-mediated linear amplification by *in vitro* transcription. Amino-allyl UTP nucleotides were incorporated for subsequent dye conjugation steps. Typically 2-5 µg aRNA was produced from one round of amplification. aRNA was quantified using Nanodrop ND-1000 and was evaluated for size distribution using Agilent RNA 6000 Nano Labchips. Pooled reference RNA was amplified once, collected and frozen in 10 µg aliquots for subsequent procedures.

5. Amplified RNA fluorescent dye conjugation

Amplified RNA was dried using a vacuum dryer at low heat setting. RNA was reconstituted in 9 µl coupling buffer (from the MessageAmp kit). Mono-functional NHS ester Cy3 or Cy5 dye, reconstituted in 11 µl DMSO, was added (Amersham Cy™Dye Post-labelling Reactive Pack, GE Healthcare, UK). In all experiments, reference aRNA and sample aRNA were labeled with Cy3 and Cy5 fluorescent dyes, respectively. Samples were incubated in dark for 30 minutes at room temperature. Hydroxylamine (4.5 µl 4M) was added for 15 minutes to quench the dye coupling reaction. Dye-coupled

RNA was purified into nuclease-free water using the aRNA filter cartridges from the MessageAmp kit in order to remove excess dye. Final volume of dye-conjugated RNA was 150 µl.

6. Microarray hybridization

A reference design was used where samples were labeled with Cy5 and amplified RNA from pig common reference RNA was labeled with Cy3. Pig common reference RNA consisted of aRNA amplified from endothelial total RNA which was pooled from all arterial sites in the study. Sample hybridizations were done in batches of 12 in random order over several days.

Samples were vacuum dried to 27 µl at low heat setting. In order to facilitate hybridization efficiency, samples were fragmented using fragmentation reagents (Ambion, Cat# 8740, Austin, TX). 3 µl 10X fragmentation reagent was added and the samples were incubated at 70 ^{0}C for 15 minutes. The fragmentation reaction was inhibited by the addition of 3 µl stop reagent. Each Cy5-conjugated sample was combined with an equal volume of Cy3-conjugated reference RNA. Nuclease-free water was added to each sample to a total volume of 70 µl followed by the addition of 1 µl of 10 mg/ml herring sperm DNA. 71 µl of 2X hybridization buffer (Proteomics Research Solutions, PRS-16003050) was added to the samples and they were incubated at 95 ^{0}C for 5 minutes. Later, they were centrifuged at 10,000g for 1 minute. Samples were loaded onto custom-printed porcine oligo microarrays. Microarrays were hybridized in a Genomics Solution HybStation (Ann Arbor, MI) using a step-down protocol (42 ^{0}C, 35^{0}C, 30^{0}C each for 5 hours). Microarrays were later washed with medium stringency buffer (PRS-16004001, Proteomics Research Solutions, Ann Arbor, MI) at 30^{0}C for 2 minutes, followed by a high stringency buffer (PRS-16004501, Proteomics Research Solutions, Ann Arbor, MI) wash at 25 ^{0}C for 2 minutes. Finally, microarrays were washed with post wash buffer (PRS-16003501, Proteomics Research Solutions, Ann Arbor, MI) at 25 ^{0}C for 2 minutes. They were later dipped into deionized water for 30 seconds and were dried by centrifugation at 500 g for 1 minute.

7. Microarray scanning and image analysis

Microarrays were scanned with an Agilent DNA Microarray Scanner at 5 µm resolution (single pass) with 100% laser power and 100% photo multiplier tube sensitivity. Images were analyzed with Agilent Feature Extraction Software (version 9.1) with raw fluorescence intensity values determined using the "CookieCutter" method of spot analysis. Each .TIF image file was examined for the quality of hybridization. If artifacts, such as uneven hybridization, were present, those microarrays were discarded. GAL file grid was fitted by hand for each microarray image to ensure correct alignment for each spot.

8. Porcine Microarray

Porcine oligonucleotide microarrays were printed at the University of Pennsylvania Microarray Core Facility using Qiagen's Pig Array-Ready Oligo Set on 16.94 mm x 52.94 mm Codelink slides. This set includes 70-mer probes for 10,665 genes from The Institute for Genomic Research (TIGR) porcine database. The average melting temperature for the primers is 78^{0}C. They were designed to have minimal hairpin structure and cross-hybridization. They are 3' biased (within 1000 base pairs of the 3' end) to ensure adequate signal tolerating some RNA degradation. Several control Cy3 spots, Stratagene alien controls and 133 custom 70-mer probes including genes known to play key roles in endothelial function and in atherosclerosis were also printed onto the microarrays. Oligos were suspended in 50 mM sodium phosphate buffer at a final concentration of 8.33 µM for printing. The resulting array has 12,288 spots arranged in 32 subgrids, each with 17 rows and 22 columns.

Several sequencing projects have contributed to the sequencing of the porcine transcriptome in recent years[3]. Frequent updating of the porcine expressed sequence tags (ESTs) necessitated the most recent annotation of the 70mers used in printing the microarrays for subsequent bioinformatics analyses. Each of the printed 70mers are derived from 64,746 Tentative Clusters (TCs) that have been built from 575,730 ESTs and 6,854 expressed transcripts from a total of 257 cDNA libraries (Porcine Gene Index: SsGI Release 12.0; June 20, 2006: Dana Farber Cancer Institute; http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pig). Using the 70mer sequences, a text file in FASTA format was created to

store the printed oligomer sequence information. In order to obtain the most recent annotation; first, these sequences were compared to the available 1,185 porcine Reference Sequence Collection (RefSeq) from the National Center for Biotechnology Information (NCBI) using the Basic Alignment Search Tool (BLAST) with a required match of 64 bases with 94% identity. As a result, 830 printed oligomers were annotated with porcine RefSeq IDs. Second, the same sequences were compared to human RefSeqs with a stringency of blast p value less than 0.001. This resulted in the annotation of 4,180 printed oligomers with human RefSeq IDs. Third, 70mers were translated into peptide sequences using "blastx" in all 6 possible reading frames and the resulting peptide sequences were compared to UniProt100 database, which contains the translation of coding sequences of multiple genomic databases. Matches with blast p value of less than 0.001 were retained. Since UniProt100 contains information about multiple species, a word comparison script was used to collapse the matches preferentially to porcine, human, mouse and rat species for a total of 4,111 UniProt IDs. In rare instances, matches for other species were allowed if porcine, human, mouse or rat were not available. Finally, similar to step three, the TC sequences (instead of the 70mers) were translated into peptide sequences using "blastx" in all 6 possible reading frames and the resulting peptide sequences were compared to UniProt100. Using these approaches, 8,962 of the 10,798 printed oligomers were fully annotated.

Functional annotation of the microarray was achieved by mapping the Uniprot IDs to Gene Ontology (GO) IDs. Gene Ontology describes gene products based on their associated biological processes, cellular components and molecular functions in a species-independent manner. Microarray spots mapped to 3,153 unique GO biological process, 1,885 GO molecular function and 599 GO cellular component IDs.

Information about the microarray and its full annotation can be found with accession number A-CBIL-16 at ArrayExpress http://www.ebi.ac.uk/microarray-as/ae/

9. Microarray Data Analysis

Microarray data for this study and all the relative MIAME compliant annotation have been deposited into the public repository ArrayExpress (accession number E-CBIL-42) through the RAD-ArrayExpress pipeline. The data and MIAME compliant annotation have also been deposited in RAD[4] and can be queried through the user-friendly RAD query interface at www.cbil.upenn.edu/RAD (RAD study_id=3265).

9a. Microarray data preprocessing

1490 spots which corresponded to control spots (blanks, Cy3, and Strategene Alien) were filtered out. For each assay, saturated spots in at least one channel were set to NA. For each channel, the mean signal measure from the Agilent Feature Extraction software was used as input signal intensity. No background subtraction was performed. M and A values were calculated from the raw data using equations 2.1 and 2.2, where *R* and *G* are the signals of the Cy5 (red) and Cy3 (green) channels, respectively. The M values were normalized with print-tip loess normalization using the Bioconductor *marray* package (version 1.12.0) for R (version 2.4.0).

9b. Analysis of differential gene expression

Differential expression analysis was performed using Patterns of Gene Expression (PaGE version 5.1.6) (http://www.cbil.upen.edu/PaGE) [5]. PaGE is a false discovery rate (FDR) based method of controlling the false positives. It uses a permutation based algorithm to estimate the FDR. In PaGE, for any specified constant, permutations of the data matrix are used to estimate the rate of false positives in any set of genes having a T-statistic greater than the constant. An appropriate constant is chosen to guarantee the desired FDR, in our case 0.25 or 0.05. Confidences are then assigned to all genes in the set. For example, if 100 genes are discovered as differentially expressed with a confidence of 0.75 (i.e. FDR of 0.25), the expected number of false positives is approximately 25. PaGE also produces "levels" of differential expression, based on the confidence parameter. Detailed information about the PaGE algorithm can be found at http://www.cbil.upenn.edu/PaGE/doc/PaGE_documentation_technical_manual.pdf . Although PaGE

identifies differentially expressed genes at computed confidence levels, genes that are not included in the differentially expressed list cannot be ruled out as not differentially expressed.

Differentially expressed genes in various comparisons were obtained using processed M values as input to PaGE. For various comparisons, genes discovered by running PaGE with an FDR of 5% or 25% were considered to be differentially expressed.

9c. Identification of enriched biological themes

Differentially expressed genes were interrogated for over-represented biological themes using Database for Annotation Visualization and Integrated Discovery (DAVID) and based on Gene Ontology (GO) terms[6]. The DAVID Functional Annotation Clustering tool was used to highlight the most relevant GO terms associated with differentially expressed gene lists[7]. Details of DAVID algorithm can be found at http://david.abcc.ncifcrf.gov/

9d. Gene Set Enrichment Analysis

Gene Set Enrichment Analysis (GSEA) is an algorithm that performs differential expression analysis at the level of gene sets[8]. The input to GSEA consists of a collection of gene sets and microarray expression data with replicates for two conditions to be compared. GSEA employs a permutation based test which uses Kolmogorov-Smirnov running sum statistic to determine which of the gene sets from the collection are differentially expressed between the two conditions. GSEA differs from differential gene expression analysis in the sense that it might identify genes which are part of a differentially expressed set but which might not be identified as significantly differentially expressed alone. The details of the GSEA algorithm can be found at http://www.broad.mit.edu/gsea/

For gene set enrichment analysis of various comparisons, e.g. athero-susceptible vs. athero-protected within coronary arteries, gene sets were created using GO mapping of the microarray. 548 GO biological process (BP), 256 GO molecular function (MF), and 142 GO cellular component (CC) gene sets were used for the study. Gene sets were made up of 15 to 500 genes. For each comparison, GSEA was performed separately for BP, MF and CC gene sets. Gene sets discovered with and FDR of 25% were considered to be significantly enriched.

9e. Ingenuity Pathway Analysis

Differentially expressed genes were analyzed for interactions with each other and other molecules using Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, www.ingenuity.com) [1]. A list of differentially expressed genes with human, mouse or rat UniProt identifiers with their corresponding confidence and expression values was uploaded into the IPA application. An *FDR* cutoff of 0.25 was set to identify genes whose expression was significantly differentially regulated. Details of the IPA algorithm can be found at http://www.ingenuity.com/library/index.html

10. Endothelial Gene Expression by Quantitative Real Time PCR

cDNA was reverse transcribed from approximately 0.5 to 1 μg total endothelial RNA with Superscript™ III Reverse Transcriptase (Invitrogen, Carlbad, CA) and was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Quantitative real time PCR (QRT-PCR) was performed using LightCycler FastStart DNA Master SYBR Green I on a LightCycler Carousel-based System (version 1.0) (Roche Applied Science, Indianapolis, IN). 2 μl sample was used in 20 μl reaction volume. Primers for each gene are listed in Supplementary Table 4. Magnesium concentrations of 3, 4 or 5 mM was optimized for each gene prior to the run. Cycling conditions were 95°C for 10 minutes followed by 35 cycles of 95 °C for 10 seconds, 55-64 °C (depending on the GC content of the primer) for 5 seconds, 72°C for 10-18 seconds (depending on the length of the PCR product). Melting curve analysis was performed at 95°C for each run to identify the amplification of a single PCR product. Gene copy numbers for each sample was obtained using the standard curves generated for each gene. For each sample, gene expression was normalized to GAPDH expression. For paired samples of aortic arch (AA) and descending thoracic aorta (DT) and renal branch (RB) and renal artery (RA) the ratio of their normalized values was calculated. Samples were paired based on their animal origin. 6-10 samples for each region were used in the analysis. Statistical significance was

assessed with one sided one-sample Wilcoxon test.

11. Endothelial protein extraction and Western blots

Freshly isolated endothelial cell scrapes were transferred directly into 50 µl ice-cold RIPA buffer (Millipore, Lake Placid, NY) which contained 1 mM EDTA, protease inhibitors: 1 mM AEBSF, hydrochloride, 0.8 µM bovine lung aprotinin, 50 µM bestatin, 15 µM E-64 protease inhibitor, 20 µM hemisulfate leupeptin, 10 µM pepstain A; phosphatase inhibitors: 11 mM sodium fluoride, 1.2 mM sodium orthovanadate, 2 mM imidazole, 1.15mM sodium molybdate, 4mM dehydrate sodium tartrate, 2mM decahydrate sodium pyrophosphate, 2 mM β-glycerophosphate (Calbiochem, San Diego, CA); and proteasome inhibitor: 10 µM MG-132 (Sigma Aldrich, St. Louis, MO). Cytoplasmic and nuclear extracts were obtained using endothelial samples pooled from 10 animals. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, catalog number 7833) were used according to manufacturer's protocols. Cells were collected in cytoplasmic extraction reagent I (CERI) containing the afore-mentioned protease and proteasome inhibitors.

Lysates were transported to the laboratory on dry ice and stored at -80°C. Cell lysates were thawed on ice. They were sonicated with three 10 second pulses to dissociate the cell membrane and proteins associated with nucleic acids. They were centrifuged at 10,000 g for 45 minutes at 4°C. The supernatants were transferred to clean ice-chilled tubes. 2.5 µl supernatant was used to measure protein concentration with BCA protein assay according to manufacturer's instructions (Thermo Scientific, Rockford, IL). β-mercaptoethanol (to a final concentration of 10% v/v), 4X NuPage LDS sample buffer (Invitrogen, Carlsbad, CA) and water (when necessary) were added to adjust the final protein concentration to 1 mg/mL.

Gel electrophoresis and membrane transfer were carried out using Invitrogen XCell II minigel system. 10 µg samples were loaded to each gel. Low molecular weight proteins (<60kDa) were separated using 4-12% Bis-Tris precast gels; and large molecular weight proteins (>60kDa) were separated using 3-8% Tris-acetate precast gels at a constant voltage of 125 volts (Invitrogen). Proteins were transferred to PVDF membranes with 0.45 micron pore size under 30V constant voltage for 1 hour (small proteins) or 2 hours (large proteins).

Membranes were incubated in 5% (w/v) fat-free dry milk prepared in Tris buffered saline with 0.05% Tween 20 (TBS-T) for 1 hour at room temperature. Primary antibodies (1:150 in 5% milk) were added onto the membranes and membranes were incubated overnight at 4°C with gentle agitation. Primary antibodies were: mouse anti-BiP/GRP78 (BD Transduction Laboratories, 610979), goat anti-XBP1 (C-20; sc-32138), rabbit anti-phospho PERK (Thr 981; sc-32577), rabbit anti-ATF6α (H-280; sc-22799), goat anti-CREB-2 (C-19; sc-7583), goat anti-phospho eIF2α (Ser 52; sc-12412), rabbit anti-PERK (H-300; sc-13073), goat anti-IRE1α (C-17; sc-10510), goat anti- GADD 153 (R-20, sc-793) all from Santa Cruz Biotechnology; rabbit anti-phospho IRE1 (S724; Abcam, ab48187), mouse anti-Lamin B1 (Zymed Laboratories, 33-2000) and mouse anti-β-actin (Sigma). They were rinsed in TBS-T 4 times for 10 minutes each. Horse radish peroxidase conjugate secondary antibody (1:5,000) and anti-biotin (1:10,000) diluted in 5% milk was added and the membrane was incubated for 1 hour at room temperature. After 4 washes with TBS-T, chemilumiscence was measured with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) according to manufacturer's instructions using FujiFilm Las-3000 image detection system (Quansys Biosciences, Logan, UT) at high sensitivity setting.

Protein bands were quantified using Multi Gauge software (version 3.0, FujiFilm). For each sample, protein expression was normalized to β-actin expression. Protein expression ratios were calculated for 10-12 paired samples of aortic arch (AA) and descending thoracic aorta (DT). Samples were paired according to animal origin. Statistical significance was assessed with one sided one-sample Wilcoxon test.

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