Supporting Information

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SI Methods

Tissue Collection. Tissues were obtained from adult pigs (6-mo-old; ~250 lb) immediately after euthanization at a local abattoir (Hatfield Industries). Ascending and descending aortas including renal arteries were harvested, and the vessel lumen was rinsed with ice-cold RNase-free PBS. Surrounding tissue was dissected, and vessels were cut open longitudinally with artery scissors to prevent damage to endothelial cells. Vessels were pinned onto waxed trays and rinsed once again with RNase-free PBS. Endothelial cells (EC) were freshly harvested by gentle scraping of regions located at (*i*) the inner curvature of the aortic arch (AA) and nearby descending thoracic aorta (DT) and (*ii*) the cranial wall and caudal wall of the aorto-renal branch and the distal renal artery. Endothelial purity assessed for AA and DT was routinely between 96 and 100% with only occasional contamination by isolated smooth muscle or leukocytic cells, and there was no significant difference between the regions. Cells were transferred directly to lysis buffer for RNA or protein extraction.

MicroRNA Microarray. The complete Minimum Information about a Microarray Experiment (MIAME)-compliant annotated study of the miRNA microarray experiments has been deposited in the public repository ArrayExpress. Information can be found with accession number E-CBIL-46 at http://www.ebi.ac.uk/microarray-as/ae/. Briefly, endothelial cells were collected from athero-susceptible AA and athero-protected dorsal DT of 35 animals. For each arterial site, endothelial samples were pooled from five animals to provide seven biological replicates for microarray experiments. Small RNAs (<200 nucleotides) were isolated using the mirVana miRNA Isolation kit (Ambion) and labeled with fluorescent Alexa Fluor dyes (aortic arch: Alex Fluor 3; descending thoracic aorta: Mexa Fluor 5) using the NCode miRNA labeling system according to the manufacturer's instructions (Invitrogen). Labeled miRNAs were hybridized to the NCode multispecies miRNA microarray (Invitrogen) overnight at 52 °C for single-color experiments. Washed microarrays were scanned with an Agilent DNA Microarray Scanner (Agilent Technologies), and the images were extracted with Agilent Feature Extraction Software (v. 9.1). The raw data were filtered by removal of control spots (dye markers, blank, empty, Alexa 5 test, and NCode controls); the foreground mean signal measures from Agilent were used to compute M values which were normalized using a print-tip loess algorithm from the Bioconductor marray (v. 1.10.0) package for R (v. 2.3.1) (1). Diagnostic plots were generated and led to retained data from only some of the print-tips (namely, those corresponding to zebrafish, rat, and one of the two human miRNA sets). Finally, reversed, shuffled, and mutated sequence reporters were removed as well as reporters for each assay with A values less than 7.5. Differential expression analysis was performed with Patterns of Gene Expression (PaGE, v. 5.1.6) (2) (http://www.cbil.upenn.edu/PaGE) to identify statistically significant differentially expressed reporters at 95% confidence [equivalent to a 5% false discovery rate (FDR)].

MicroRNA Quantitative Real-Time PCR. Expression of selected miRNAs was quantified by two-step quantitative real-time PCR using the TaqMan miRNA reverse transcription kit, TaqMan miRNA assay kits (Applied Biosystems), and the LightCycler System (Roche Applied Science) according to the manufacturers' instructions. Individual miRNA expression was normalized in relation to expression of small nuclear U6B RNA, a commonly used internal control for miRNA quantification assay (3).

In Situ Hybridization. Expression of miR-10a and miR-10b in swine aorta and freshly isolated porcine endothelial cells was detected by a modified FISH assay developed by Silahtaroglu et al. (4). The method combines the unique miRNA recognition properties of locked nucleic acid (LNA)-modified oligonucleotide probes with FISH using tyramide signal amplification (TSA) technology. LNA-modified oligonucleotide probes targeting miR-10a and miR-10b were purchased from Exiqon and labeled with digoxigenin (DIG) using the DIG Oligonucleotide Tailing Kit (Roche Applied Science). Isolated arterial porcine endothelial cells were fixed onto microscope slides; intact swine aorta was frozen in Tissue-Tek OCT compound. Isolated endothelial cells or fixed 10-μm cryostat sections of frozen swine aortas were hybridized with LNA-modified oligonucleotide probes targeting miR-10a or miR-10b at 53 °C for 1 h. The slides were washed and incubated with HRP-conjugated anti-DIG antibody (Roche Applied Science) for 30 min at room temperature, followed by FITC–tyramide signal amplification (PerkinElmer) for 10 min at room temperature in the dark. The slides were spin-dried and mounted with ProLong Gold (Invitrogen) with DAPI on glass coverslips. Images were acquired and analyzed using a Zeiss fluorescence microscope with Axiovision image analysis software.

cDNA Quantitative Real-Time PCR. Quantitative real-time PCR was performed using LightCycler FastStart DNA Master SYBR Green I or Master^{plus} SYBR Green I on a LightCycler System (Roche Applied Science). PCR primers for genes of interest are listed in Table S1. For in vitro samples, the gene expression was normalized to human ubiquitin expression. For in vivo isolated EC, gene expression was normalized to the geometric mean of expression of swine ubiquitin, GAPDH, and platelet/endothelial cell adhesion molecule 1(PECAM-1).

Human Whole-Genome Microarray. The complete MIAME-compliant annotated study of the human microarray experiment has been deposited in the public repository ArrayExpress. Information can be found with accession number E-CBIL-45 at http://www.ebi.ac.uk/microarray-as/ae/. Briefly, low-passage human aortic endothelial cells (HAECs) were transfected with 50 nM *Homo sapiens* miR-10a (hsa-miR-10a) miRIDIAN hairpin inhibitor or miRIDIAN microRNA hairpin inhibitor negative control (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen) in 60-mm Petri dishes. Total RNAs were isolated by mirVana miRNA Isolation kit (Ambion) 48 h after transfection and were assessed for quality using an Agilent Bioanalyzer 2100 (Agilent Technologies). Two-color microarrays with a dye-swap design that reverses the dye assignments in control and miR-10a knockdown experiments were used to mitigate the systemic dye bias (5). Total RNAs were isolated from eight paired replicates consisting of a 60-mm Petri dish of control HAECs and a dish of miR-10a knockdown HAECs. Cyanine-3- or Cyanine-5-CTP–labeled cRNAs were generated from ~200 ng total RNA using the Agilent Low RNA Input Liner Amplification Kit (Agilent Technologies) and then were hybridized to Agilent whole human genome 4 × 44K microarrays according to the manufacturer's instructions. To achieve dye swap, four microarrays were hybridized with Cyanine-3–conjugated cRNAs from control cells and Cyanine-5–conjugated cRNAs from miR-10a knockdown cells, and the reverse was done in the other four arrays. Microarrays were hybridized at 65 °C for 17 h, washed, and scanned at 5-μm resolution (single pass) with 10% and 100% photo multiplier tube sensitivity with an Agilent DNA Microarray Scanner. Images were extracted and analyzed with Agilent Feature Extraction Software

(v. 9.1). The Agilent foreground mean signal measures were used to generate M values which were normalized by global loess, after filtering control spots, using the Bioconductor marray (v. 1.20.0) package for R (v. 2.8.1) (1). Differential expression analysis was performed using PaGE (v. 5.1.6) (http://www.cbil.upenn.edu/PaGE) (2). The FDR was set at 5%, so 95% of the predicted genes are expected to be true positives.

Gene Set Enrichment Analysis. Gene set enrichment analysis (GSEA) is an analytical algorithm that interprets gene expression data at the level of gene sets, that is, groups of genes sharing common biological function, chromosomal location, or regulation (6). It determines statistically whether a priori-defined gene sets show concordant differences between two biological states and has been shown to identify successfully biological pathways and processes that characterize the distinct cellular phenotypes. GSEA employs a permutation-based test that uses Kolmogorov-Smirnov running sum statistic to determine which gene sets from the collection are expressed differentially in the two conditions. The details of the GSEA algorithm can be found at http://www.broad.mit.edu/gsea/. We used 724 Gene Ontology (GO) biological processes for the study. Gene sets discovered with an FDR of 25% were considered significantly enriched.

Luciferase Reporter Assay. miRNA/target duplexes and putative miR-10a binding elements in the MAP kinase kinase kinase 7 (MAP3P7) and β-transducin repeat-containing gene (βTRC) 3′ UTR were predicted by RNAhybrid (7). Three tandem repeats of these binding elements were synthesized and cloned into the luciferase 3′ UTR of a pRL-TK vector which constitutively expresses Renilla luciferase (Promega), as shown in Fig. S5. (pRL-TK expression vector with luciferase 3′ UTR inserted with putative let-7b binding sites was a generous gift from Zissimos Mourelatos.) Low-passage (<4) HEK 293 cells were cotransfected in 24-well plates using Lipofectamine LTX (Invitrogen) with a given pRL-TK vector (0.5 μg) and the control pGL3 vector (0.5 μg) that expresses Firefly luciferase. The cells were transfected 24 h later with 1 nm hsa-miR-10a mimics or miRNA mimic negative controls (Dharmacon) using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). Firefly and Renilla luciferase activities were measured consecutively by an EnVision 2103 multilabel plate reader (PerkinElmer), using the dual luciferase assays (Promega) 24 h after the transfection of hsa-miR-10a mimics or miRNA mimic negative controls. Renilla luciferase values were normalized to firefly luciferase values for vector normalization.

Endothelial Protein Extraction and Western Blots. Protocols of endothelial protein isolation and Western blots were described previously (8). Briefly, HAECs in a 60-mm Petri dish and fresh endothelial scrapes from a single animal were collected in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Millipore) containing protease inhibitors, phosphatase inhibitors, and proteasome inhibitors. Total protein was isolated by sonication and centrifugation of the cell lysates and measured by bicinchoninic acid protein assay (Pierce).

Cytoplasmic and nuclear extracts of the endothelial protein were isolated by NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific). For the in vivo isolated porcine aortic endothelial cells, proteins were extracted from endothelial samples pooled from six animals and later were concentrated using a vacuum dryer at low-heat setting (Thermo Fisher Scientific).

Gel electrophoresis and membrane transfers were carried out using the XCell II mini-gel system (Invitrogen). Samples (10–20 μg) were loaded into wells of a 4–12% Bis-Tris precast gel (Invitrogen) that separates the proteins at a constant voltage of 100 V. Proteins were transferred to 0.45-μm PVDF membranes which were incubated sequentially with primary antibodies (Table S2) and HRP-conjugated secondary antibodies. Secondary antibodies were purchased from Santa Cruz Biotechnology and Pierce. Chemiluminescence measurements were performed using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and FujiFilm Las-3000 image detection system (Quansys Biosciences). Protein bands were quantified using MultiGauge software, v. 3.0 (FujiFilm). Total and cytoplasmic protein expression was normalized to β-actin expression. Nuclear protein expression was normalized to nuclear lamin B1 expression.

1. Yang YH, et al. (2002) Normalization for cDNA microarray data: A robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* 30:e15.
2. Grant GR, Liu J, Stoeckert CJ, Jr (2005) A practical false discovery rate approach to identifying patterns of differential expression in microarray data. *Bioinformatics* 21: 2684–2690.
3. Chen C, et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 33:e179.
4. Silahtaroglu AN, et al. (2007) Detection of microRNAs in frozen tissue sections by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification. *Nat Protoc* 2:2520–2528.
5. Yang YH, Speed T (2002) Design issues for cDNA microarray experiments. *Nat Rev Genet* 3:579–588.
6. Subramanian A, et al. (2005) Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* 102:15545–15550.
7. Kruger J, Rehmsmeier M (2006) RNAhybrid: MicroRNA target prediction easy, fast and flexible. *Nucleic Acids Res* 34 (Web Server issue):W451–454.
8. Civelek M, Manduchi E, Riley RJ, Stoeckert CJ, Jr, Davies PF (2009) Chronic endoplasmic reticulum stress activates unfolded protein response in arterial endothelium in regions of susceptibility to atherosclerosis. *Circ Res* 105:453–461.

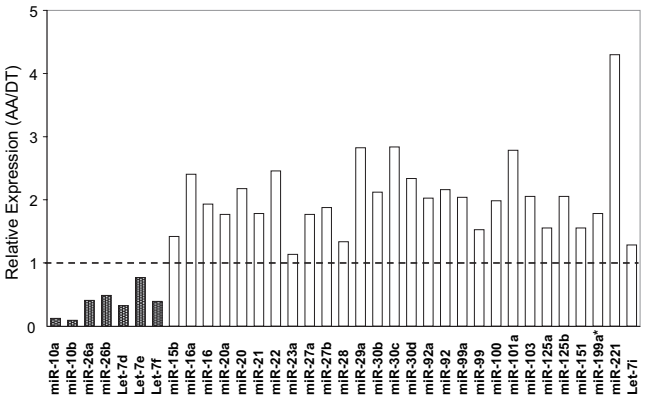

Fig. S1. Relative expression of 35 endothelial miRNAs in swine AA and nearby DT identified by miRNA microarrays using permutation-based PaGE analysis with an FDR of 5%.

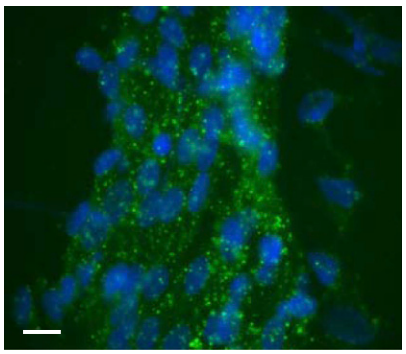

Fig. S2. *En face* endothelial expression of miR-10a (green). Endothelium taken directly from the arterial surface (DT) was fixed onto microscope slides. miR-10a expression was detected by FITC using LNA-FISH (1). Nuclei were observed by DAPI staining (blue). (Scale bar, 20 μM.)

1. Silahtaroglu AN, et al. (2007) Detection of microRNAs in frozen tissue sections by fluorescence in situ hybridization using locked nucleic acid probes and tyramide signal amplification. *Nat Protoc* 2:2520–2528.

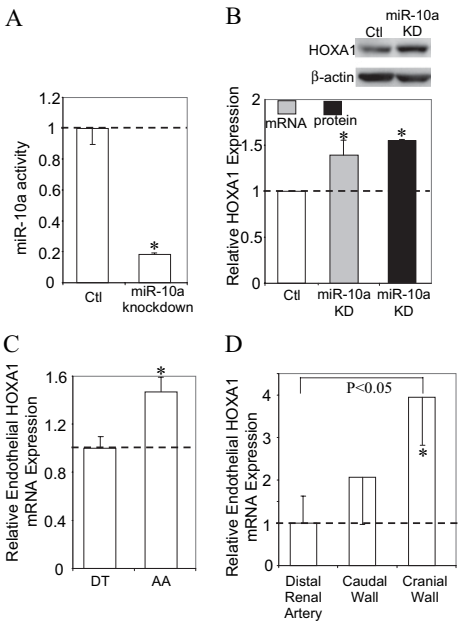

Fig. S3. Increased expression of transcription factor Homeobox A1 (HOXA1) by miR-10a and up-regulation of endothelial HOXA1 in athero-susceptible arterial regions. (*A*) Suppression of endogenous miR-10a in HAEC by miR-10a inhibitors (n = 8 paired samples); U6B RNA was used as internal control (Ctl). (*B*) Up-regulation of HOXA1 mRNA and protein in miR-10a-knockdown (KD) HAEC. Gene (n = 8) and protein (n = 3) expression was normalized to ubiquitin and β-actin, respectively. (*C*) Elevated endothelial expression of HOXA1 mRNA in the AA compared with nearby DT (n = 10). (*D*) Increased endothelial expression of HoxA1 mRNA in the athero-susceptible cranial regions of the aorto-renal branches compared with the athero-protective distal renal artery (n = 3 paired samples; each sample was pooled from four animals). Data represent means ± SEM. $^*P < 0.05$.

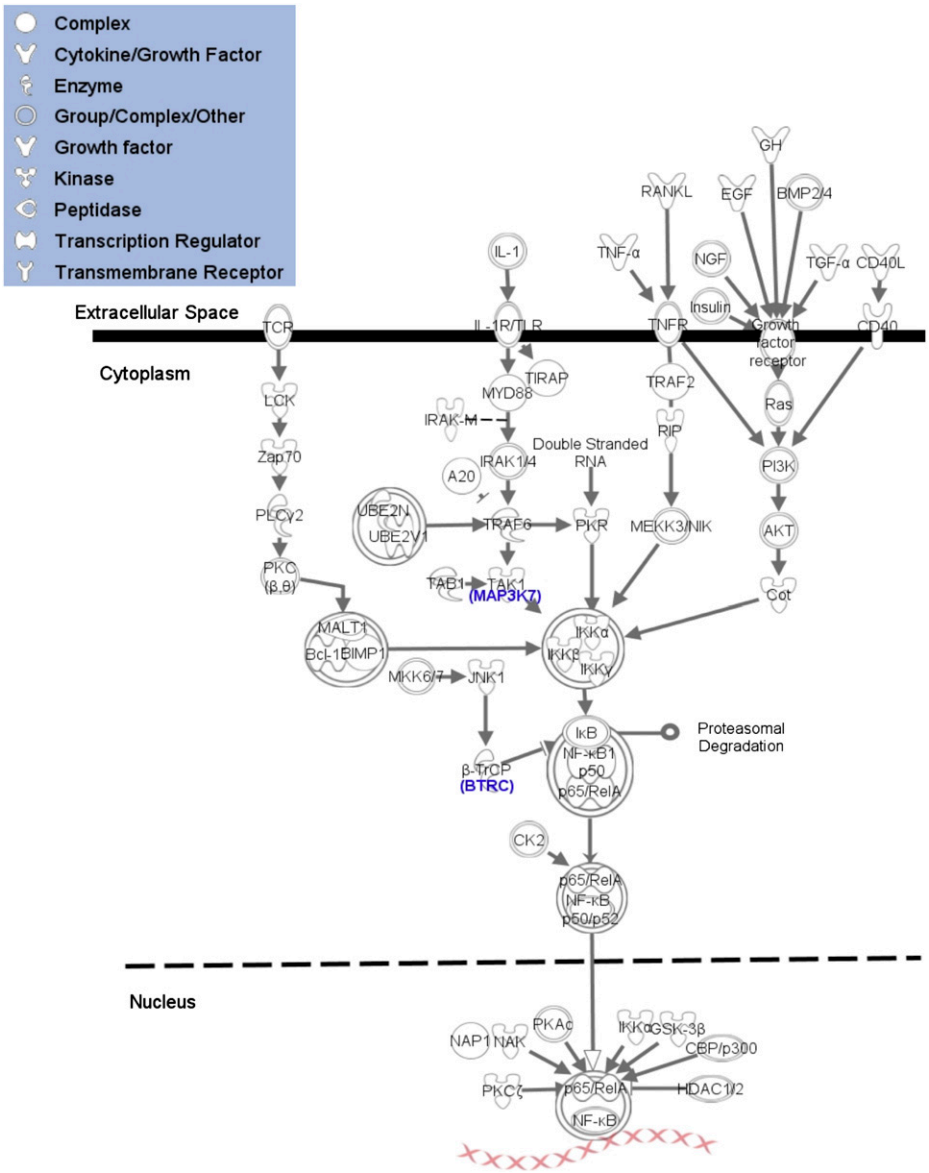

Fig. S4. Canonical pathway of NF-κB signal transduction generated by the Ingenuity Pathway Analysis (IPA) software. The adjacent key demonstrates the shapes that identify each molecular category in IPA Pathways.

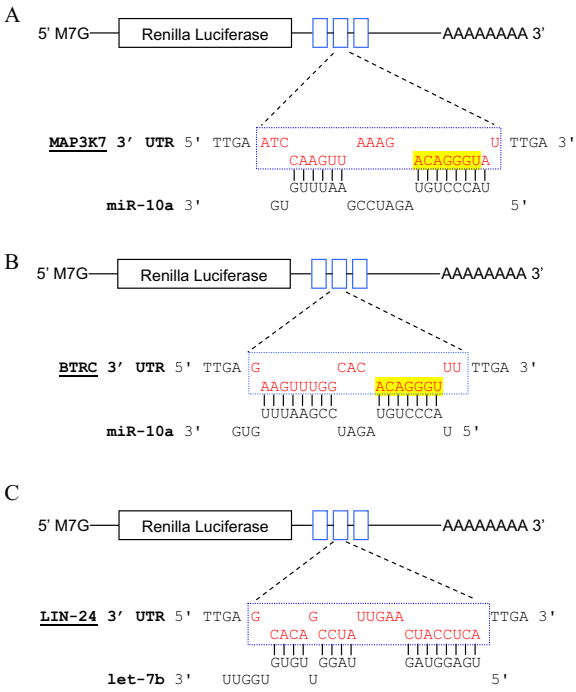

Fig. S5. Schematic representation of the luciferase vectors containing miR-10a recognition elements cloned from (*A*) MAP3K7 3′ UTR, (*B*) βTRC 3′ UTR, and (*C*) the luciferase construct incorporated with the let-7b recognition element cloned from LIN-24. Nucleotides in red represent the cloned miRNA binding elements predicted by RNAhybrid (1). Yellow boxes indicate the putative binding sites for the miR-10a seed region.

1. Kruger J, Rehmsmeier M (2006) RNAhybrid: MicroRNA target prediction easy, fast and flexible. *Nucleic Acids Res* 34 (Web Server issue):W451–W454.

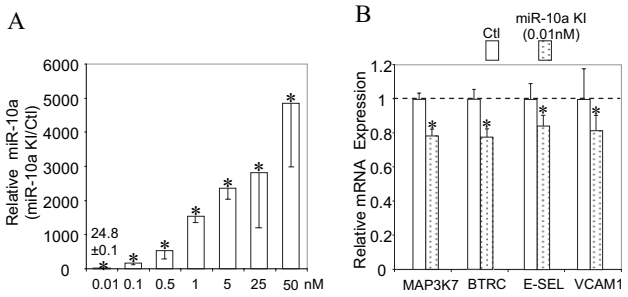

Fig. S6. Suppression of MAP3K7, βTRC, and proinflammatory adhesion molecules by miR-10a overexpression in HAEC. (*A*) Dose-dependent miR-10a overexpression in HAEC. Titration of miR-10a mimics (0.01–50 nm) resulted in a dose-dependent knockin of mature miR-10a in HAECs (n = 3–8). Ctl, control. (*B*) A physiologically relevant level of miR-10a overexpression (~25-fold) suppressed gene expression of endothelial MAP3K7, βTRC, E-selectin, and vascular cell adhesion molecule 1 (VCAM-1) (n = 6–11). Data represent mean ± SEM. *$P < 0.05$.

Table S1. PCR primer sequences and annealing conditions

Gene	Primer sequences	Annealing temperature (°C)
Human		
Ubiquitin	Forward: GAGGTGGAGCCCAGTGACA Reverse: ATGTTGTAGTCAGAAAGAGTGCGG	62
HOXA1	Forward: CCAGGAGCTCAGGAAGAAGAGAT Reverse: CCCTCTGAGGCATCTGATTGGGTTT	57
SELE	Forward:TGTGGTTGAGTGTGATGCTGTGA Reverse: TTGCAGGATGATTTGAAGGTGAAC	61
VCAM-1	Forward:GATACAACCGTCTTGGTCAG Reverse: TAATTCCTTCACATAAATAAACCC	57
IL-6	Forward:GCCTTCGGTCCAGTTGCCTT Reverse: GCAGAATGAGATGAGTTGTC	61
IL-8	Forward:TGTAGGGTTGCCAGATG Reverse: GCAGACTAGGGTTGCC	59
MCP-1	Forward:CCAGCAGCAAGTGTCCCAAAG Reverse: TGCTTGTCCAGGTGGTCCATG	59
MAP3K7	Forward:ACTTGATGCGGTACTTTC Reverse: GGTTGCGGCGATCCTA	60
βTRC	Forward:AGATTAGTGCAGCCCG Reverse: GGAGCTAAGAGCGCCA	59
Swine		
Ubiquitin	Forward: TGACCAGCAGCCTCTGATT Reverse:TCTTGTCGCAGTTGTATTTCTGAG	64
GAPDH	Forward:GGGCGATGCTGGTGCTGAGTATGT Reverse:ACGTTGGCAGTAGGGACACGGAAG	63
PECAM-1	Forward:CCTCGCCCATTTCCTACCAACTTT Reverse:CAGACTCCACCTCCTCGCTCAG	63
HOXA1	Forward:CAGGACCCAAACGTAT Reverse: GTGTGGTACTTCGGGA	57

Table S2. Antibodies and applications

Antibody	Source/epitope	Application	Dilution	Commercial source
β-actin	Mouse monoclonal anti-frog	Human	1:10000	#ab6276, Abcam
		Swine	1:10000	
IκBα	Rabbit monoclonal anti-human	Human	1:5,000	#ab5682, Abcam
		Swine	1:5,000	
Phospho-IκBα	Rabbit polyclonal anti-human	Human	1:2,000	#ab32518, Abcam
		Swine	1:2,000	
p65	Rabbit polyclonal anti-human	Human	1:100	#RB-1638, Thermo Scientific
		Swine	1:100	
Lamin B1	Mouse monoclonal anti-chicken	Human	1:500	#33–2000, Invitrogen
		Swine	1:500	
MAP3K7	Goat polyclonal anti-human	Human	1:500	#ab50431, Abcam
		Swine	1:500	
βTRC	Goat polyclonal anti-human	Human	1:200	#sc-8862, Santa Cruz
		Swine	1:200	
HOXA1	Goat polyclonal anti-human	Human	1:200	#sc-17146, Santa Cruz