ORIGINAL RESEARCH

Abnormal Endothelial Gene Expression Associated With Early Coronary Atherosclerosis

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BACKGROUND: We examined feasibility of a unique approach towards gaining insight into heritable risk for early atherosclerosis: surveying gene expression by endothelial cells from living subjects.

METHODS AND RESULTS: Subjects aged <50 years (mean age, 37; range, 22–49) without obstructive coronary artery disease underwent coronary reactivity testing that identified them as having normal or abnormal coronary endothelial function. Cultures of Blood Outgrowth Endothelial Cells (BOEC) from 6 normal and 13 abnormal subjects passed rigorous quality control and were used for microarray assessment of gene expression. Of 9 genes differentially expressed at false discovery rate <0.1%, we here focus upon abnormal subjects having elevated expression of HMGB1 (high mobility group box 1) which we unexpectedly found to be linked to low LAMC1 (laminin gamma 1) expression. This linkage was corroborated by 3 of our past studies and confirmed bio-functionally. Compared with normal BOEC, abnormal BOEC released 13±3-fold more HMGB1 in response to lipopolysaccharide; and they deposited one tenth as much LAMC1 into collagen subendothelial matrix during culture. Clinical follow-up data are provided for 4 normal subjects (followed 13.4±0.1 year) and for 12 abnormal subjects (followed 9.1±4.5 years).

CONCLUSIONS: The known pathogenic effects of high-HMGB1 and low-LAMC1 predict that the combination would biologically converge upon the focal adhesion complex, to the detriment of endothelial shear responsiveness. This gene expression pattern may comprise a heritable risk state that promotes early coronary atherosclerosis. If so, the testing could be applied even in childhood, enabling early intervention. This approach offers a way to bridge the information gap between genetics and clinical phenotype.

Key Words: atherosclerosis ■ endothelial function ■ focal adhesion complex ■ focal adhesion kinase ■ HMGB1 ■ laminin ■ risk factor ■ shear stress

Clinical atherosclerosis emerges from complexity involving multiple promotive influences, cell types, and biologic systems. Although heritable factors are believed to account for ≥50% of disease risk, only rarely does this involve a single gene exerting a large influence. Rather, the heritable component of risk most likely involves multiple genes that individually exert smaller effects. Identifying these has been a formidable challenge. We here demonstrate the feasibility of using a unique approach to bridging the information gap between genomics and clinical phenotype: assessing gene expression by endothelial cells obtained from living patients.

For this we use blood outgrowth endothelial cells (BOEC) from cultures of peripheral blood. Unlike cell types often labeled “EPC,” BOEC are fully differentiated, bona fide endothelial cells that are the in vitro progeny of a circulating, marrow-derived, transplantable endothelial progenitor. Importantly, BOEC themselves have never been exposed to
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CLINICAL PERSPECTIVE

What Is New?
- Using subjects aged <50 years shown by clinically-indicated coronary reactivity testing to have either normal or abnormal coronary endothelial function, this feasibility study surveyed gene expression by blood outgrowth endothelial cells (a stable endothelial type that can identify heritable differences in gene expression) to bridge the gap between genomics and clinical phenotype—and has thereby provisionally identified a heritable risk factor for early development of atherosclerosis.
- Compared with blood outgrowth endothelial cells from subjects having normal coronary endothelial function, blood outgrowth endothelial cells from subjects with abnormal coronary endothelial function exhibited abnormally elevated expression of HMGB1 (high mobility group box 1) that was linked to abnormally depressed expression of LAMC1 (laminin gamma 1).

What Are the Clinical Implications?
- This combination may be a heritable risk factor for early atherosclerosis, since known effects and functions of HMGB1 and LAMC1 predict that their abnormal expression in the observed directions would biologically converge at the focal adhesion complex and endothelial cell membrane in a manner detrimental to the vascular endothelial cell’s normal responsiveness to shear stress.

Methods

This gene expression study was done in 2005 to 2007 and, hence, reflects technologies then extant. The delay in submission for publication was because the project leader paused from work for a decade because of a family medical catastrophe.

Methods described herein are sufficient to enable replication of the study. The new gene expression data underlying this report are deposited and available at Gene Expression Omnibus, series GSE132651; previously reported data extracted for use herein were previously deposited as series GSE22688 and GSE9877. Aliquots of the BOEC samples studied herein are present in our BOEC bio-bank and may be accessible by contacting the corresponding author.

Subjects

This study was approved by the Institutional Review Boards at the University of Minnesota and the Mayo Clinic. Subjects were adults, aged <50 years (all but 1 was <46 years), undergoing clinically indicated invasive angiography (at the Mayo Clinic Catheterization Laboratory) for signs and/or symptoms suggestive of angina plus risk factors. Subjects gave written informed consent.

Coronary Reactivity Testing

Patients withheld all vasoactive prescription medications for at least 24 to 48 hours, and fasted for 12 hours, before coronary angiography and coronary reactivity testing.8,10 Following diagnostic angiography and exclusion of obstructive coronary artery disease, we positioned a Doppler guidewire (Flowire, Volcano Therapeutics Inc, Rancho Cordova, CA) within a coronary-infusion catheter into the mid-left anterior descending (LAD) coronary artery.11,12 We gave escalating intracoronary doses of acetylcholine (10⁻⁶, 10⁻⁵, and 10⁻⁴ mol/L for 3 minutes at each concentration), infused selectively into the mid-LAD. We measured coronary artery diameter offline (by an independent investigator) in the segment 5 mm distal to the tip of the Doppler wire, using a quantitative

inflammatory or tissue-specific influences of the in vivo environment. Even at high-fold expansion they are far more stable than other endothelial types, and they are generic endothelial reporter cells uniquely suitable for a study such as this.3 Since they can be obtained from a known donor, BOEC enable matching of donor characteristics to endothelial features of interest, in the context of measured heritable gene expression. Indeed, we previously applied this approach to identify underlying risk for a clinical stroke phenotype affecting some children with sickle cell anemia4 and, separately, to suggest an influence of ancestral continent-of-origin on endothelial shear stress responsiveness.5
coronary angiography program (Medis Corp, Leiden, the Netherlands) as previously described. Coronary blood flow in the LAD from the Doppler derived time velocity integral and vessel diameter, where coronary blood flow = \( \pi \times \text{coronary artery diameter}^2 \times \text{average peak velocity}/2 \). Coronary endothelial dysfunction was defined as >20% decrease in mid-LAD diameter and/or <50% increase in coronary blood flow in response to acetylcholine infusion. Subjects were thus identified as having normal or abnormal coronary endothelial dysfunction. These data for individual subjects are summarized in Table S1.

BOEC Culture

At time of angiography, before heparin administration, we drew 50- to 100-mL venous blood into citrate anti-coagulant and sent it to the University of Minnesota. Within 4 hours of venipuncture, we established BOEC cultures using an updated version of the long-term culture method we originally developed. Using Histopaque-1077 and blood diluted with Ca++/Mg++ free Hanks buffered salt solution, we obtained blood mononuclear cells. These were washed twice with BOEC culture medium (EBM2 basal medium plus EGM-2 SingleQuot and 10% fetal bovine serum) and resuspended in 4 mL of the same. All 4 mL of cell suspension was added to a single well of a 6-well culture plate previously coated with collagen I. Cultures were incubated at 37°C in a humidified environment having 5% CO₂. After 16 hours, a gentle wash with culture medium removed debris and unattached cells. Thereafter, culture medium was changed daily for 7 days and thereafter on every other day. Subcultures were established on collagen I whenever cells reached 60% to 70% confluence. An important aspect of the method is fastidious application and meticulous performance of each of the extraordinary precautions we originally adopted (Data S1).

We harvested cells at a nominal 10⁶-fold expansion to collect \( \approx 3 \times 10^7 \) BOEC. This degree of expansion falls well within a broad safe expansion window wherein (deliberately induced) acquired effects have washed out, yet long before onset of gene expression instability. For the resulting 28 unique-patient BOEC cultures, we used fresh cells for quality control and RNA preparation, and we cryopreserved aliquots for later experimental use.

Nineteen BOEC cultures passed our multi-parameter quality control testing requiring: cobblestone morphology; staining positive for VE-cadherin, von Willebrand Factor, and P1H12 (CD146); staining negative for CD133 and CD14; a single population of cells at the sensitivity of light microscopy; and normal cytogenetics. Our previous studies documented that cultures meeting all these criteria additionally: are negative for CD133 and positive for multiple additional endothelial antigens; are 100% endothelial by FACS; display typical endothelial features such as VCAM-1 upregulation in response to tumor necrosis factor/interleukin-1, uptake of acetylated low-density lipoprotein, and tube formation in Matrigel; exhibit presence of Weibel Palade bodies; and display endothelial lineage fidelity by gene expression. Each of the 9 quality control failures was because of cytogenetics analysis returned as being abnormal for culture acquired abnormalities. This left us with BOEC from the 6 normal and 13 abnormal subjects reported herein (Table 1).

Gene Expression

From each culture we isolated total RNA that was then reverse transcribed, quality verified, labeled, fragmented, and applied to Affymetrix U133A microarrays (assay for 14 500 well characterized genes and 18 400 transcripts/variants). To minimize possible batch effects, all samples for gene expression were profiled in a single batch at the University of Minnesota Microarray Core facility. As previously described, we used the robust multi-array average method to background-adjust, quantile-normalize, and summarize expression using median polish algorithm, as implemented in the software Genedata Expressionist Pro3.1PP (Basel, Switzerland). Our analysis applied the R function “t test” for the Welch t test (we report uncorrected \( P \) values) and the R package “samr” for Significance Analysis of Microarrays that reports false discovery rate (FDR) with 500 permutations and a delta value of 0.719; the code

Table 1. Subjects at Time of Enrollment

|                      | Normal (n=6)       | Abnormal (n=13)       |
|----------------------|--------------------|-----------------------|
| Age, y               | 36.8±10.4 (24–49)  | 37.4±6.1 (22–45)      |
| BMI, kg/m²           | 23.5±4.1 (20.1–31.0)| 27.7±3.9 (22.8–35.2) |
| C-reactive protein, nmol/L | 4±1 (3–6)       | 18±23 (1–76)          |
| C-reactive protein, mg/L | 0.4±0.1 (0.3–0.6) | 1.8±2.3 (0.1–7.6)     |
| Men                  | 2/6                | 7/13                  |
| White                | 6/6                | 13/13                 |
| Hypertension         | 0/6                | 2/13                  |
| Diabetes mellitus    | 0/6                | 0/13                  |
| Hyperlipidemia       | 0/6                | 10/13                 |
| Smoker, active       | 2/6                | 3/13                  |
| Smoker, never        | 3/6                | 7/13                  |
| Family history positive | 3/6              | 11/13                 |
| History chest pain   | 6/6                | 13/13                 |

BMI indicates body mass index.
Hierarchical Clustering

We conducted 2 clustering analyses, using only the universe of 43 transcripts exhibiting differential expression at $P<0.001$, and 1 using the universe of 9 transcripts exhibiting FDR $<0.1%$. We used R function "hclust" for unsupervised hierarchical clustering, using normalized gene expression, complete linkage, and 1 minus Pearson correlation as the distance measure. Expression level per probe was centered and normalized to have variance 1 before clustering. To assess relative discriminatory importance amongst these 43 transcripts, we constructed a random forest using normal/abnormal expression ratio as the response and each of the 43 transcripts as predictors. We determined variable importance by the mean decrease in the Gini coefficient in R package “randomForest.”

Informatics

To identify biological inter-relationships we searched using databases: Enrichr, Ensembl, DIANA-TarBase v.8, miRBase, PathwayCommons, genomatix, Ingenuity Pathway Analysis, and the cardiovascular literature. BOEC gene expression data for the present 19 study subjects have been deposited in the NCBI Gene Expression Omnibus repository with accession number GSE132651 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132651). Other data sets used herein were deposited in the past: GSE22688 and GSE9877.

Bio-Functional Validation Testing

From cryopreserved aliquots, we re-established BOEC cultures from normal and abnormal subjects to seek bio-functional confirmatory data.

To assay HMGB1 (high mobility group box 1) content of culture medium, we used $1.9\times10^5$ BOEC at 85% confluence, switched to serum-free medium, and incubated $100 \text{ng/mL lipopolysaccharide}$. After 24 hours an ELISA measured HMGB1 released into medium.

To assay LAMC1 (laminin gamma 1) deposition into subendothelial matrix, we plated BOEC onto collagen I coated wells in sufficient number to reach confluence in 2 days. We then maintained them for 14 days, changing culture medium 3 times a week. Then, after rinsing with PBS, we treated culture wells with 0.5% Triton for 10 minutes to eliminate cell bodies but leave behind extracellular matrix on the well. An in situ ELISA measured LAMC1 in matrix.

RESULTS

Characteristics of the 6 normal and 13 abnormal study subjects at time of coronary and gene studies reveal that, on average, the abnormal subjects had somewhat higher body mass index, C-reactive protein, and risk factor burden (Table 1). All but one subject was aged $<46$ years.

The reporter cells used here, BOEC, have themselves never been exposed to in vivo signaling effects. Rather, they are the in vitro outgrowth progeny of a circulating, marrow-derived progenitor cell. Further, the necessary degree of their expansion in culture is sufficient for (deliberately) induced inflammatory responses to fully wash out yet is still many logs below the expansion degree at which phenotypic or gene expression drift is seen. Unlike other endothelial cell types, BOECs are very stable.

Single Gene Expression

At the significance threshold of FDR $<10\%$, 29 transcripts exhibited differential expression for abnormal versus normal subjects (Table 2). Among the 9 transcripts exhibiting differential expression at the highly stringent threshold of FDR $<0.1\%$, there was little overlap in the expression value ranges for normal versus abnormal groups (Figure 1).

Focus on HMGB1

Of the identified genes, we herein focus on the increased expression of $HMGB1$ by normals (1.4-fold, FDR $<0.1\%$, $P=5.7\times10^{-5}$) because of this protein’s known and prominent role in arterial disease pathogenesis, specifically including the biology of atherosclerosis, per Discussion. Arguing that high $HMGB1$ expression amongst abnormal subjects is not reflective of any proinflammatory in vivo milieu, $HMGB1$ elevation was not accompanied by differential expression by any of 40 other inflammatory genes (Table S3).

Inverse Expression Linkage

Inspection of individual subject expression values suggested a sub-cluster of 7 abnormal with highest $HMGB1$ expression and a sub-cluster of 7 abnormal with lowest $LAMC1$ expression (Figure 1). These 2 sub-clusters were composed, nearly perfectly, of the same subjects, and there was a strong inverse correlation
between HMGB1 and LAMC1 expression for the 19 study subjects (Figure 2A).

Providing indirect corroboration of this unexpected observation, we uncovered the same high-HMGB1/low-LAMC1 relationship in our past studies of BOEC gene expression for separate groups of: 38 healthy 20- to 29-year-olds (Figure 2B),5 27 random normal subjects (Figure 2C),4 and 20 children with sickle cell anemia (Figure 2D).4 There was no apparent effect of sex on this high-HMGB1/low-LAMC1 relationship.

Hierarchical Clustering
Hierarchical clustering analysis using the universe of 43 transcripts exhibiting differential expression at P<0.001 generated 2 primary clusters, one containing all normals and the other containing all abnormalities (Figure S1A). The latter cluster had 2 secondary sub-clusters that separate the abnormalities having highest versus lowest HMGB1 expression. This suggests that HMGB1 expression is an important—but not sole—discriminator.

Indeed, a random forest analysis of the same transcript universe estimated the strongest discriminators to also include MCT4 (SLC16A3), RABGGTB, LAMC1, UBE2G2, POLR2C, PNO1, and HMGCS1 (Figure S2). That these help discriminate between lowest- versus highest-HMGB1 expressers (regardless of subject group) was supported by another hierarchical clustering analysis using only the 9 transcripts having FDR <0.1% (Figure S1B). This accurately generated 2 primary clusters: 1 composed of those abnormal subjects having the highest HMGB1 expression, and the other containing those abnormalities having the lowest HMGB1 expression plus all normals.

Table 2. Differentially Expressed Transcripts at Threshold of False Discovery Rate <10%, Listed in Order of False Discovery Rate and Then by P Value

| Probe Set | Gene | False Discovery Rate (%) | P Value | FOLD (Abnormal/ Normal) | NAME |
|-----------|------|---------------------------|---------|-------------------------|------|
| 209041_s_at | UBE2G2 | ≤0.1 | 2.9×10−6 | 1.28 | Ubiquitin conjugating enzyme E2 G2 |
| 209181_s_at | RABGGTB | ≤0.1 | 8.5×10−6 | 1.27 | Rab geranylgeranyltransferase subunit beta |
| 203622_s_at | PNO1 | ≤0.1 | 1.3×10−5 | 1.42 | Partner of NOB1 homolog |
| 202855_s_at | SLC16A3 | ≤0.1 | 2.0×10−5 | 1.84 | Solute carrier family 16 member 3 (MCT4) |
| 208996_s_at | POLR2C | ≤0.1 | 4.3×10−5 | 1.34 | RNA polymerase II, subunit C |
| 214938_x_at | HMGB1 | ≤0.1 | 5.7×10−5 | 1.40 | High mobility group box 1 |
| 212714_at | LARP4 | ≤0.1 | 1.1×10−4 | 1.20 | La ribonucleoprotein 4 |
| 213825_at | Olig2 | ≤0.1 | 1.3×10−4 | 1.11 | Oligodendrocyte transcription factor 2 |
| 219082_at | AMDHD2 | ≤0.1 | 1.3×10−4 | 0.82 | Amidohydrolase domain containing 2 |
| 218447_at | CMC2 | 6.75 | 3.2×10−5 | 1.35 | C-X9-C containing motif containing 2 |
| 220890_s_at | DDX47 | 6.75 | 4.1×10−5 | 1.22 | DEAD box helicase 47 |
| 216149_at | LRRRC7BP1 | 6.75 | 5.7×10−5 | 1.13 | Leucine rich repeat containing 37B pseudogene 1 |
| 220016_at | AHNAK | 6.75 | 2.3×10−4 | 1.15 | AHNAK nucleoprotein |
| 211999_at | H3F3B | 6.75 | 3.7×10−4 | 1.24 | H3 histone family member 3B |
| 208672_at | SFRS3 | 6.75 | 5.7×10−4 | 1.30 | Serine and arginine rich splicing factor 3 |
| 212394_at | EMC1 | 6.75 | 6.1×10−4 | 1.13 | ER membrane protein complex subunit 1 |
| 202856_s_at | MCT4 | 6.75 | 7.9×10−4 | 1.72 | Solute carrier family 16 member 3 |
| 200700_s_at | KDELR2 | 6.75 | 8.1×10−4 | 1.17 | KDEL endoplasmic reticulum protein retention receptor 2 |
| 201574_at | ETF1 | 6.75 | 8.2×10−4 | 1.22 | Eukaryotic translation termination factor 1 |
| 201862_s_at | LRRFIP1 | 6.75 | 9.7×10−4 | 1.49 | LRR binding FLII interacting protein 1 |
| 207094_at | IL8RA | 6.75 | 1.3×10−3 | 1.11 | C-X-C motif chemokine receptor 2 |
| 214058_s_at | MYCL1 | 6.75 | 1.6×10−3 | 1.11 | MYCL proto-oncogene, bHLH transcription factor |
| 201862_s_at | LRRFIP1 | 6.75 | 1.8×10−3 | 1.49 | LRR binding FLII interacting protein 1 |
| 218948_at | ORSL1 | 6.75 | 3.3×10−3 | 1.26 | Glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1 |
| 216302_at | HNRPC | 6.75 | 3.9×10−3 | 1.11 | Heterogeneous nuclear ribonucleoprotein C (C1/C2) |
| 202564_x_at | SNX15 | 6.64 | 1.1×10−4 | 0.86 | Sorting nexin 15 |
| 200770_s_at | LAMC1 | 6.64 | 1.5×10−4 | 0.71 | Laminin subunit gamma 1 |
| 214150_x_at | ATP6V0E | 6.64 | 4.2×10−4 | 0.80 | ATPase H+ transporting V0 subunit e1 |
| 221097_s_at | KONM2 | 6.64 | 5.8×10−3 | 1.08 | K+ Ca-activated channel subfamily regulatory beta subunit |
Bio-Functional Validation

To bio-functionally test for elevated HMGB1 protein, we measured its release from BOEC incubated with lipopolysaccharide. Consistent with their abnormally high HMGB1 gene expression, BOEC from abnormal released 13 ±3-fold greater HMGB1 than normal BOEC released; \( P=8.5 \times 10^{-5} \) (Figure 3, left). This provides bio-functional confirmation that higher HMGB1 gene expression by abnormal subjects is real.

To bio-functionally test for lowered LAMC1 protein, we incubated BOEC on plates originally coated with collagen and measured LAMC1 deposition into the subendothelial matrix after 14 days. Consistent with their abnormally depressed LAMC1 expression, BOEC from abnormals deposited, on average, one tenth as much LAMC1 as identically-incubated normal BOEC (Figure 3, right). Despite the discernable difference, the result from this test was not statistically significant, likely because of small sample size.

Clinical Follow-Up

Since this clinical study was performed in 2005 to 2007, follow-up data are available but incomplete (institutional review board rules prohibit contacting non-Mayo subjects from this closed study) (Table 3). For 4 normals followed for 13.4±0.1 years, 1 experienced a major adverse cardiovascular event. For 12 abnormals followed for 9.1±4.5 years, 4 of 12 developed major adverse cardiovascular events. Interestingly, of these 4 abnormals who developed major adverse cardiovascular events, 3 were among the top-4 highest HMGB1 expressors.

DISCUSSION

In addressing risk for early coronary endothelial dysfunction, the earliest clinical form of atherosclerosis, we surveyed gene expression by Blood Outgrowth Endothelial Cells as an approach to bridging the troublesome gap between genetics and clinical phenotype. Our operational definition of “early” was subject age <50 years, and coronary reactivity testing identified subjects as having normal or abnormal coronary endothelial function. Our results indicate that the approach is, indeed, feasible and possibly can shed light upon underlying risk. Comparing abnormals versus normals we identified differential expression of 29 transcripts at FDR <10%,
of which 9 were significant at the stringent threshold of FDR <0.1%.

Of the latter group, we here focus on the abnormalities having elevated expression of HMGB1 in apparent linkage with lowered expression of LAMC1. We first review the most relevant aspects of HMGB1 and LAMC1 proteins, as each has unambiguous implications for atherogenesis. Then we focus on their expected gene-gene interactions that predict a remarkable, pathobiological convergence that would jeopardize endothelial cell responsiveness to shear stress.

**HMGB1**

HMGB1 expression was greater for abnormal subjects (1.4-fold, FDR <0.1%, \( P = 5.7 \times 10^{-5} \)), and the ranges for normal versus abnormal subjects barely overlapped. HMGB1, an “alarmin”, is one of the damage-associated molecular pattern molecules. Its roles are diverse, remarkable, and highly relevant to atherosclerosis.20-22

**Nucleus**

HMGB1 is the most abundant non-histone nuclear protein, and it regulates multiple nuclear functions, among them gene expression. For example, HMGB1 enhances binding of nuclear factor-kB p50/p50 and p65/p50 to DNA, and it is reportedly required for p50 to be functional.23 Although it traffics bidirectionally across the nuclear membrane, HMGB1 is normally highly skewed towards the nucleus. In monocytes, however, the skew is heavily towards cytoplasm.24

**Cytoplasm**

In cytoplasm, HMGB1 is part of many regulatory protein complexes, and it promotes translocation of nuclear factor-kB, RelA and SP1 into the nucleus. Notably, HMGB1 associates with cytoplasmic Src, exerting an inhibitory influence that is discussed in the Gene-Gene Interactome section below.

**Export**

During necrosis or in response to injury or stimuli, HMGB1 is passively or actively exported from various cell types.20-22 For example, endothelial cells release it in response to tumor necrosis factor,25 abnormal shear stress,26 or hypoxia,20 Smooth muscle cells release it in response to cholesterol.27 Neutrophils disgorge it into neutrophil extracellular traps,28 and platelets contribute it to thrombi.29 Monocytes/macrophages release HMGB1 in response to inflammatory stimuli and when manifesting their inflammatory reprogramming.

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**Figure 2.** Inverse correlation between high-HMGB1 (high mobility group box 1) and low-LAMC1 (laminin gamma 1) expression. Scales display expression units which differ amongst panels because each study was done in a different time frame. A, The present study in which normal are in open symbols and abnormal in closed symbols, \( r = -0.844 \). B, Healthy 20- to 29-year-olds, \( r = -0.569 \). C, Random 23- to 69-year-olds, \( r = -0.728 \). D, Children with sickle cell anemia, \( r = -0.383 \). Gene expression data are deposited at Gene Expression Omnibus, series GSE132651, GSE22688, GSE9877, GSE9877, respectively.
Extracellular

Upon release, HMGB1 can act as a cytokine or chemokine, able to induce and amplify inflammation by engaging multiple receptors, especially toll-like receptor 4 and the receptor for advanced glycation end-products. Indeed, HMGB1 is a dominant driver of sterile inflammation. It activates monocytes/macrophages and platelets, as well as endothelial and smooth muscle cells that then adopt a proliferative phenotype. It causes endothelial barrier hyperpermeability, facilitating egress of WBC and atherogenic lipid, and it activates endothelial NADPH oxidase.

Atherogenesis

Each of the above HMGB1 effects is atheroprotective in nature and would plausibly be exaggerated in those with inherently high HMGB1 expression.

SNP Insight

We did not conduct SNP testing here, but extant data link increased HMGB1 expression to arterial disease. The HMGB1 3814 polymorphism (rs2249825) is predicted to create a strong enhancer effect on HMGB1 expression, and leukocytes from such individuals actually do exhibit exaggerated HMGB1 release when challenged with lipopolysaccharide—just as we saw here for BOEC from abnormals. Studied in Chinese, this SNP is associated with hypertension, increased cerebral ischemia size, and exaggerated inflammation in sepsis.Indeed, HMGB1 already is believed to promote all stages of atherosclerosis from inception to plaque rupture, and it is specifically implicated in coronary, peripheral and cerebral arterial disease. For example, antibody-mediated neutralization of HMGB1 attenuated atherosclerosis by >50% in apolipoprotein E deficient mice, and genetic deletion or neutralization of HMGB1 prevented intimal hyperplasia in response to carotid wire injury.

Table 3. Available Subject Data at Subsequent Clinical Follow-Up

|                      | Coronary Endothelial Function |
|----------------------|------------------------------|
|                      | Normal | Abnormal |
| Number available     | 4/6    | 12/13    |
| Age at coronary study, y | 38.0±10.4 (24–49) | 40.8±1.6 (38–42) |
| Follow-up duration, y | 13.4±0.1 (13.3–13.5) | 9.1±4.5 (1.2–14.0) |
| Experienced MACE, n  | 1/4    | 4/12     |
| Age at MACE, y       | 55     | 50±6 (39–54) |
| Study-to-MACE interval, y | 13     | 1, 12, 12, 12 |

For the normal subject this was a myocardial infarction. For the abnormal subjects it was: 1 new diagnosis of congestive heart failure; 1 stroke; 1 myocardial infarction; 1 peripheral artery disease and carotid endarterectomy needed. MACE indicates major adverse cardiovascular events.
the laminin γ1 chain. Indeed, experimental knockout of LAMC1 resulted in embryonic lethality with failure to make basement membranes. Remarkably, laminin α5β1γ1 was experimentally found to be necessary for endothelial response to shear stress.

Thus, even if LAMC1 production is just lowered, harmful consequences may be predictable. One is enhanced endothelial barrier hyperpermeability. Another consequence is that in endothelial basement membranes if laminin is missing it is maladaptively replaced with fibronectin, replacing laminin-enforced endothelial quiescence with fibronectin-mediated inflammatory signaling. Particularly notable is that endothelial cells on laminin are shear responsive, but those on fibronectin are not. Thus, depressed LAMC1 production could replicate several key features of atherogenesis.

Atherogenesis
We find nothing in the literature that directly links LAMC1 to atherosclerosis, but there are some suggestive data. First, a study of quantitative trait loci identified the Ath44 region of chromosome 1 as being associated with aortic root lesion size in murine atherosclerosis; LAMC1 is a gene in this region. Second, an analysis of bio-functional pathways enriched in advanced versus early coronary atherosclerosis identified focal adhesion (critical in shear responsiveness) to be an implicated functional module, within which LAMC1 was one of the abnormally downregulated genes associated with arteriopathy severity.

Gene-Gene Interactome Impacting Endothelial Function
For all these reasons, we predict that the combination of high-HMGB1 plus low-LAMC1 expression in endothelium would converge biologically and detrimentally at the focal adhesion complex that is required for endothelial cell mechanosensing of shear stress. Specifically, focal adhesion complex function would be impaired by HMGB1 because it inhibits the reciprocal phosphorylations between Src and FAK (focal adhesion kinase) that enable focal adhesion complex to participate in normal shear sensing. As for LAMC1, it is necessary for the proper basement membrane engagement with endothelial abluminal integrins that is required for their clustering, an on-switch for focal adhesion complex function. That could be jeopardized if LAMC1 production is low.

We suggest that these known effects of high-HMGB1 and of low-LAMC1 would synergistically undermine the endothelial cell's ability to respond adaptively and optimally to shear stress. Thus, we hypothesize that impairment of shear responsiveness in this manner would maladaptively foster endothelial dysfunction and, thereby, earlier development of detectable atherogenesis.

Concordance of High-HMGB1 and Low-LAMC1 Expression
The unexpected linkage between high-HMGB1 and low-LAMC1 expression revealed by our data is corroborated via archived data sets from our 3 previous studies of BOEC gene expression (Figure 2). This suggests an underlying regulatory relationship, although our data cannot inform as to mechanism. The primary aberrancy could be high-HMGB1, or low-LAMC1, or something else affecting both. So, to illustrate relevance but simplify discussion, we here arbitrarily assume that elevated HMGB1 expression is the primary aberrancy that drives lower LAMC1 expression.

Suppression of LAMC1 is possible via microRNA (miR) species already implicated in atherosclerosis. Perhaps most intriguing is miR-21 that is induced by both HMGB1 and oscillatory flow regimes, and miR-21 is known to target LAMC1. Interestingly, miR-21 is the most abundant miR in normal BOEC (Hebbel and Steer, unpublished observation, 2012), and it is upregulated in human atherosclerotic tissue. Other LAMC1-targeting miRNAs are identified, as well as some that target LAMC1 transcription factors. One of these is ESR1 (estrogen receptor alpha) that not only is hypermethylated in atherosclerosis but also is a target of miR-206, another miR that can be induced by HMGB1.

Caveats and Limitations
This study probes the early onset of atherosclerosis, but we recognize that "early" here can mean truly earlier onset and/or accelerated progression and/or earlier symptom awareness. The gene expression changes we have highlighted are perhaps consistent with each. Regardless, we surmise that such changes would not only nudge an individual's homeostatic balance in a maladaptive direction but also enhance susceptibility to risk factors. For example, elevated constitutive HMGB1 expression could result in its exaggerated release from endothelium residing in anatomically atheroprone areas.

We emphasize that all of our subjects were studied by coronary catheterization because they had an episode of chest pain. Thus, our normal subjects were not truly normal individuals. Rather, our labels “normal” and “abnormal” refer specifically to their coronary endothelial function status. Consistent with this, our abnormal subjects did exhibit somewhat greater...
frequency of some risk factors, most notably hyperlipidemia and elevated C-reactive protein. Experimentally, HMGBl can induce C-reactive protein, but hyperlipidemia can stimulate HMGBl release of HMGBl. We find no evidence that C-reactive protein or lipid increase HMGBl expression.

Cultured cells always raise concerns about laboratory-induced variations. However, our prior standardization, reproducibility, and validation studies plus our extensive experience with BOECs, justify confidence that our data identify true differences between endothelial cells from abnormal versus normal. We emphasize that a critical factor in achieving this is the fastidious application of all our extraordinary culture precautions described in Data S1.

Further, measured BOEC gene expression is not influenced by in vivo signaling exposures as the BOEC themselves have never been exposed to inflammation or tissue-specific signaling. Their 10th-fold expansion is logs beyond what is needed for inflammatory signaling effects to wash out, and it is logs before any phenotypic or genotypic drift appears. BOEC are far more stable in culture than other endothelial types. Thus, we believe that here, as in our previous studies of this nature, results likely reflect heritable differences between abnormal and normal subjects.

Of course, it is impossible to absolutely exclude the possibility that, in the abnormal subjects, an atherogenic environment created durable epigenetic changes within the circulating endothelial progenitors, changes then passed to their outgrowth progeny, the BOEC. Somewhat mitigating this concern, elevated HMGBl expression by the BOEC from abnormal subjects was not accompanied by differential expression of any of 40 other inflammation-responsive genes.

Finally, we do not know if these expression changes are actually endothelial specific. If not, perhaps they could be identified in a more easily accessible cell type. For other cell types, however, there would be magnified concern about acquired influences or artifacts. And, of course, if interest lies in the functional biology of endothelial cells, BOEC uniquely offer the opportunity to examine this.

CONCLUSIONS

Our data reveal an association between high-HMGBl plus low-LAMC1 expression with coronary endothelial dysfunction at age <50 years. This pathobiologically-relevant, probably-heritable combination could create risk via a detrimental biological convergence that maladaptively impairs endothelial mechanosensing. Our results may have practical clinical implications since the approach can be applied even in children, perhaps enabling identification of those who would most benefit from earlier, more aggressive medical intervention.

We recognize, of course, that this feasibility study is too limited to be definitive. Nonetheless, it does support the notion that BOEC can be used to bridge the information gap between genomics and clinical phenotype in understanding atherosclerosis risk. Indeed, BOEC comprise a unique platform that enables matching donor characteristics with endothelial functional assessment with various “omics.” In that regard, sufficient BOEC can easily be produced to enable studying all “omics” simultaneously for each endothelial cell culture. We suggest that this would be uniquely useful for achieving the truly integrative endothelial “omics” that may be key in understanding the heritable component of atherosclerosis risk.

ARTICLE INFORMATION

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Disclosures

None.

Supplementary Materials

Data S1
Tables S1–S3
Figures S1–S2

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SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS

BOEC culture

The present study was enabled by our prior developmental, standardization, and validation studies performed in 2000-2003 and described elsewhere (text references 2-5). These involved BOEC cultures from >150 unique individuals, with gene expression surveyed on >80. At our period of peak activity, we successfully established BOEC cultures 2-3 times per week, with a success rate of ~90%. As developed by the Hebbel Lab (Univ of Minnesota) our method includes taking each of the following extraordinary precautions to avoid culture variation effects and to maximize reproducibility.

   [a] Necessary supplies, reagents, microarray chips, culture medium ingredients are acquired in sufficient quantity prior to study onset so there is no risk that lot number of anything could change in mid-study.

   [b] At venipuncture, the first few ml of peripheral venous blood is discarded.

   [c] Blood is maintained at room temperature between venipuncture and culture by using special shipping boxes ⁸ that provide this protection.

   [d] Although in the past we have successfully established BOEC cultures up to 8 hours after venipuncture, we make every effort to minimize the venipuncture-to-culture delay. In the present study the interval between blood attainment and starting culture set-up was <4 hours.
[e] Any single step of the overall process (i.e., culture set-up/maintenance, quality control assessments, RNA preparation, cell biology experiments) is always performed by the same trained and highly-experienced technician known to consistently achieve highly reproducible results. For example, for the present study the single BOEC culture technician was available around-the-clock daily for ~2 years.

[f] All cultures are set up in the same culture room, using the same culture hood, using the same temperature/humidity/gas-controlled incubator. For our studies, a culture room is dedicated to BOEC specifically, and only the designated BOEC culture technician has access.

[g] All cultures are passed to the same extent, a nominal million-fold expansion providing ~3x10^7 BOEC. They are always harvested 4 hours after the last change of culture medium and when at 85-90% confluence. This degree of expansion is solidly within what we previously found to be a “safe window” of expansion: deliberately acquired gene expression changes (from IL-1/TNF) have completely washed out; and the cells are several logs of expansion shy of developing instability of phenotype or gene expression.

[h] Quality Control: All cultures are subjected to rigorous quality control measures, with success indicated by: cobblestone morphology; positive for VE-cadherin and vWF and P1H12(CD146); negative for CD45 and CD14; single population of cells at level of light microscopy. In addition, all cultures are submitted for cytogenetics analysis (to enable later exclusion of data from any exhibiting culture-acquired cytogenetic abnormalities).

Our prior standardization and validation studies revealed that BOEC cultures passing this multi-parameter screen additionally: are negative for CD133; 100%
endothelial at level of FACS; positive for multiple endothelial antigens (flk1, PECAM-1, VCAM-1, ICAM-1, CD34, CD51, thrombomodulin); exhibit typical endothelial behaviors such as “in vitro angiogenesis”, acLDL uptake, VCAM-1 upregulation in response to TNF/IL-1. Also, gene expression profiling confirms endothelial lineage identity, and EM reveals Weibel Palade bodies.

**Antibodies Used**

| Antibody          | Source     | catalog #  | Working dilution |
|-------------------|------------|------------|------------------|
| anti-vWF          | Sigma      | F3520      | 1 µg/ml          |
| anti-VE-cadherin  | Santa Cruz | sc-6458    | 1 µg/ml          |
| anti-CD146        | Hebbel lab | P1H12      | 5 µg/ml          |
| anti-CD45         | Santa-Cruz | sc-25590   | 1 µg/ml          |
| anti-CD14         | Santa Cruz | sc-9150    | 1 µg/ml          |
| anti-HMGB1        | ABCAM      | ab190377   | 1:500 dilution   |
| anti-LAMC1        | Sigma      | sab 4051727| 1 µg/ml          |

**Code used for samir:**

```r
require(samr)
x = as.matrix(expressionDat)
y = c(rep(2,13),rep(1,6))
data=list(x=x,y=y, geneid=probe.id, genenames= gene.id, logged2=F)
samr.obj<samr(data, resp.type="Two class unpaired", nperms=500)
delta.table <- samr.compute.delta.table(samr.obj)
delta=0.719
samr.plot(samr.obj,delta)
siggenes.table<-samr.compute.siggenes.table(samr.obj,delta, data, delta.table)
```
Table S1. Coronary reactivity assessment summary.

Definition(s) of abnormal coronary endothelial function:
less than 50% increase in coronary blood flow (CBF) in response to highest dose acetylcholine
and/or
more than 20% reduction in coronary artery diameter (CAD) in response to highest-dose acetylcholine

| subject | % change CBF in response to highest-dose acetylcholine | % CAD change in response to highest-dose acetylcholine | subject group assignment |
|---------|-------------------------------------------------------|------------------------------------------------------|--------------------------|
| A       | 52                                                    | -15                                                  | NL                       |
| B       | 99                                                    | -9                                                   | NL                       |
| C       | 133                                                   | 0                                                    | NL                       |
| D       | 129                                                   | 0                                                    | NL                       |
| E       | 555                                                   | 49                                                   | NL                       |
| F       | 57                                                    | -20                                                  | NL                       |
| G       | 63                                                    | -30                                                  | ABNL                     |
| H       | 20                                                    | -42                                                  | ABNL                     |
| I       | -24                                                   | -28                                                  | ABNL                     |
| J       | 56                                                    | -30                                                  | ABNL                     |
| K       | 48                                                    | 7                                                    | ABNL                     |
| L       | 7                                                     | -4                                                   | ABNL                     |
| M       | 31                                                    | -21                                                  | ABNL                     |
| N       | 46                                                    | -10                                                  | ABNL                     |
| O       | -10                                                   | -26                                                  | ABNL                     |
| P       | -24                                                   | -36                                                  | ABNL                     |
| Q       | 22                                                    | -33                                                  | ABNL                     |
| R       | -57                                                   | -35                                                  | ABNL                     |
| S       | -100                                                  | -100                                                 | ABNL                     |
Table S2. Differentially expressed transcripts at threshold of Welch $P<0.001$, listed in order of $P$ value.

| PROBE SET       | GENE      | FDR    | $P$     | FOLD (ABNL/NL) | EXPANDED NAME                                                                 |
|-----------------|-----------|--------|---------|----------------|-----------------------------------------------------------------------------|
| 209041_s_at     | UBE2G2    | ≤0.1   | 2.9x10^{-6} | 1.28           | ubiquitin conjugating enzyme E2G2                                           |
| 209181_s_at     | RABGGTB   | ≤0.1   | 8.5x10^{-6} | 1.27           | Rab geranylgeranyltransferase subunit beta                                  |
| 203622_s_at     | PNO1      | ≤0.1   | 1.3x10^{-5} | 1.42           | partner of NOB1 homolog                                                   |
| 202855_s_at     | SLC16A3   | ≤0.1   | 2.0x10^{-5} | 1.84           | solute carrier family 16 member 3                                          |
| 218447_at       | CMC2      | 6.75   | 3.2x10^{-5} | 1.35           | C-X9-C containing motif containing 2                                       |
| 220890_s_at     | DDX47     | 6.75   | 4.1x10^{-5} | 1.22           | DEAD box helicase 47                                                       |
| 208996_s_at     | POLR2C    | ≤0.1   | 4.3x10^{-5} | 1.34           | RNA polymerase II, subunit C                                               |
| 214938_x_at     | HMGB1     | ≤0.1   | 5.7x10^{-5} | 1.40           | high mobility group box 1                                                  |
| 216149_at       | LRRC37BP1 | 6.75   | 5.7x10^{-5} | 1.13           | leucine rich repeat containing 37B pseudogene 1                            |
| 212714_at       | LARP4     | ≤0.1   | 1.1x10^{-4} | 1.20           | La ribonucleoprotein domain family member 4                                |
| 202564_x_at     | SNX15     | 8.64   | 1.1x10^{-4} | 0.86           | sorting nexin 15                                                          |
| 213825_at       | OLIG2     | ≤0.1   | 1.3x10^{-4} | 1.11           | oligodendrocyte transcription factor 2                                      |
| 219082_at       | AMDHD2    | ≤0.1   | 1.3x10^{-4} | 0.82           | N-acetylglucosamine-6-phosphate deacetylase                                |
| 200770_s_at     | LAMC1     | 8.64   | 1.5x10^{-4} | 0.71           | laminin subunit gamma 1                                                   |
| 212601_at       | ZZEF1     | >10    | 1.5x10^{-4} | 0.88           | zinc finger, ZZ type with EF hand domain                                    |
| 213826_s_at     | H3F3B     | >10    | 1.8x10^{-4} | 0.84           | H3 histone, family 3B                                                      |
| 44120_at        | ADCK2     | >10    | 2.1x10^{-4} | 0.91           | aarF domain containing kinase 2                                            |
| 220016_at       | AHNAK     | 6.75   | 2.3x10^{-4} | 1.15           | AHNAK nucleoprotein                                                       |
| 203202_at       | KRR1      | >10    | 2.8x10^{-4} | 1.29           | KRR1, small subunit processsome component homolog                          |
| 208765_s_at     | HNRNPR    | >10    | 3.5x10^{-4} | 1.18           | heterogeneous nuclear ribonucleoprotein R                                  |
| 207734_at       | LAX1      | >10    | 3.7x10^{-4} | 1.08           | lymphocyte transmembrane adaptor 1                                         |
| 211999_at       | H3F3B     | 6.75   | 3.7x10^{-4} | 1.24           | H3 histone family member 3B                                               |
| 205822_s_at     | HMGCS1    | >10    | 4.0x10^{-4} | 1.48           | 3-hydroxy-3methylglutary-Coenzyme A synthase 1                             |
| 217370_x_at     | FUS       | >10    | 4.2x10^{-4} | 1.29           | FUS RNA binding protein                                                   |
| 214150_x_at     | ATP6V0E   | 8.64   | 4.2x10^{-4} | 0.80           | ATPase H+ transporting V0 subunit e1                                       |
|probe id| gene symbol| expression| fold change| description |
|--------|-------------|------------|-------------|-------------|
|221255_s_at| EMC6| >10| 4.4x10^-4| ER membrane protein complex subunit 6 |
|217370_x_at| FUS| >10| 4.2x10^-4| RNA binding protein FUS |
|222382_x_at| NUP205| >10| 5.1x10^-4| nuclear pore complex protein Nup205 |
|201965_s_at| SETX| >10| 5.7x10^-4| senataxin |
|208672_s_at| SFRS3| 6.75| 5.7x10^-4| serine and arginine rich splicing factor 3 |
|212394_at| EMC1| 6.75| 6.1x10^-4| ER membrane protein complex subunit 1 |
|219836_at| ZBED2| >10| 6.2x10^-4| zinc finger, BED-type containing 2 |
|202722_at| HMGCL| >10| 6.7x10^-4| hydroxymethylglutaryl-CoA lyase mitochondrial |
|202856_s_at| SLC16A3| 6.75| 7.0x10^-4| solute carrier family 16 member 3 |
|208990_s_at| HNRPH3| >10| 7.1x10^-4| heterogeneous nuclear ribonucleoprotein H3 (2H9) |
|211933_s_at| HNRNPA3| >10| 7.2x10^-4| heterogeneous nuclear ribonucleoprotein A3 |
|214409_at| RFPL3S| >10| 7.3x10^-4| RFPL3 antisense [ncRNA] |
|215558_at| C6orf133| >10| 8.1x10^-4| chromosome 6 open reading frame 13315 |
|204647_at| HOMER3| >10| 8.2x10^-4| homer scaffold protein 3 |
|201574_at| ETF1| 6.75| 8.2x10^-4| eukaryotic translation termination factor 1 |
|214882_s_at| SFRS2| >10| 8.7x10^-4| splicing factor, arginine-serine rich 2 |
|200700_s_at| KDELRF| 6.75| 8.7x10^-4| KDEL endoplasmic reticulum protein retention R2 |
|201862_s_at| LRRFIP1| 6.75| 9.7x10^-4| LRR binding FLII interacting protein 1 |
|210269_s_at| >10| 9.9x10^-4| 0.85| DNA segment on X & Y 155 expr. sequence |
Table S3. ABNLs vs NLs did not significantly differ in expression of 40 inflammation-response genes (for each gene, all transcripts are listed).

| Gene  | Probest      | Fold Difference | P    |
|-------|--------------|-----------------|------|
|       | ABNL/NL)     |                 |      |
| AGER  | 210081_at    | 1.00            | 0.987|
|       | 217046_s_at  | 0.87            | 0.076|
| CAT   | 215573_at    | 0.98            | 0.616|
| C3    | 217767_at    | 0.90            | 0.077|
|       | 211922_s_at  | 0.81            | 0.110|
|       | 201432_at    | 0.83            | 0.044|
| C4    | 214428_x_at  | 1.02            | 0.554|
|       | 208451_s_at  | 1.00            | 0.985|
| C5    | 205500_at    | 1.04            | 0.600|
| CCL2  | 216598_s_at  | 0.90            | 0.827|
| CCL3  | 205114_s_at  | 1.00            | 0.974|
| CRP   | 205753_at    | 0.99            | 0.885|
|       | 37020_a      | 0.94            | 0.215|
| F3    | 204363_at    | 1.00            | 0.971|
| HMOX1 | 203665_at    | 1.01            | 0.978|
| ICAM1 | 215485_s_at  | 0.87            | 0.305|
|       | 202638_s_at  | 0.73            | 0.294|
|       | 202637_s_at  | 0.84            | 0.371|
| IFNB1 | 208173_at    | 0.97            | 0.631|
| IL1β  | 205067_at    | 1.00            | 0.936|
|       | 39402_at     | 0.98            | 0.515|
| IL2   | 207849_at    | 0.98            | 0.645|
| IL6   | 205207_at    | 1.20            | 0.213|
| IL8   | 202859_x_at  | 0.90            | 0.678|
|       | 211506_s_at  | 0.68            | 0.412|
| JUN   | 213281_at    | 1.00            | 0.981|
|       | 201466_s_at  | 0.94            | 0.607|
|       | 201465_s_at  | 0.92            | 0.281|
|       | 201464_x_at  | 0.91            | 0.313|
| MYC   | 202431_s_at  | 1.09            | 0.272|
| NFKB1 | 209239_at    | 1.02            | 0.785|
| NFKB2 | 209636_at    | 0.88            | 0.089|
|       | 207535_s_at  | 0.84            | 0.309|
|       | 211524_at    | 1.04            | 0.384|
| NFKBIA| 201502_s_at  | 0.86            | 0.267|
| NOS1  | 207309_at    | 1.06            | 0.202|
|       | 207310_s_at  | 0.98            | 0.576|
| NOS3  | 205581_s_at  | 1.08            | 0.781|
| PPARG | 208510_s_at  | 0.74            | 0.154|
| PTK2  | 207821_s_at  | 0.95            | 0.514|
|       | 208820_at    | 0.92            | 0.241|
| Gene | Description | Expression | Log2 Fold Change |
|------|-------------|------------|-----------------|
| REL  |             | 206036_s_at | 0.99            |
| RELA |             | 201783_s_at | 1.01            |
| RELB |             | 205205_at   | 0.88            |
| SELE |             | 206211_at   | 0.65            |
| SEL  |             | 206049_at   | 0.91            |
| SP1  |             | 214732_at   | 1.04            |
| TGFβ |             | 203084_at   | 0.94            |
| TLR2 |             | 204924_at   | 0.79            |
| TLR4 |             | 221060_s_at | 0.81            |
| TP53 |             | 211300_s_at | 1.06            |
| TNF  |             | 207113_s_at | 1.01            |
| SOD1 |             | 200642_at   | 1.02            |
| SRC  |             | 221281_at   | 0.95            |
| SOD1 |             | 213324_at   | 0.97            |
| SRC  |             | 221284_s_at | 0.92            |
| VCAM1|             | 203868_s_at | 1.14            |
| VEGF |             | 212171_x_at | 0.95            |
|      |             | 210513_s_at | 0.91            |
|      |             | 211527_x_at | 0.87            |
|      |             | 210512_s_at | 0.84            |
|      |             | 211527_x_at | 0.87            |
|      |             | 212171_x_at | 0.95            |
Figure S1. Hierarchical clustering analyses. A. Clustering using the universe of all 43 transcripts significant at P<0.001 suggests *HMGB1* is important but not the sole discriminator between NLs vs ABNLs. The low-*HMGB1* subjects in the ABNL group are outlined in the inset. B. Clustering using only the 9 transcripts significant at FDR<0.1% reveals substructure discriminating all lowest *HMGB1* expressers (those encircled in the inset) from the highest *HMGB1* expressers, regardless of subject group.
We applied a random forest approach (using the universe of 43 transcripts significant at \(P<0.001\) that yielded the clustering pattern shown in Supplemental Figure 1A) to estimate relative degree of contribution made by individual transcripts/genes in correctly separating ABNLs from NLs. Stronger contribution is rightwards on the horizontal axis. At the left, transcripts are listed by gene names in one of three columns indicating their significance level: FDR>10% (far left), FDR<10% but >0.1% (middle), FDR <0.1% (right).