Comparative gene expression analysis between coronary arteries and internal mammary arteries identifies a role for the *TES* gene in endothelial cell functions relevant to coronary artery disease

Stephen R. Archacki[1,2,3], George Angheloiu[2], Christine S. Moravec[2,3], Hui Liu[4], Eric J. Topol[5] and Qing Kenneth Wang[1,2,3,4],*

[1]Center for Cardiovascular Genetics, Department of Molecular Cardiology, Lerner Research Institute and [2]Department of Cardiovascular Medicine, Cleveland Clinic, Cleveland, OH 44195, USA, [3]Department of Biological, Geological, and Environmental Sciences, Cleveland State University, Cleveland, OH 44115, USA, [4]Key Laboratory of Molecular Biophysics of the Ministry of Education, College of Life Science and Technology and Center for Human Genome Research Center, Huazhong University of Science and Technology, Wuhan 430074, PR China and [5]Scripps Translational Science Institute, The Scripps Research Institute, La Jolla, CA 92037, USA

Coronary artery disease (CAD) is the leading cause of death worldwide. It has been established that internal mammary arteries (IMA) are resistant to the development of atherosclerosis, whereas left anterior descending (LAD) coronary arteries are athero-prone. The contrasting properties of these two arteries provide an innovative strategy to identify the genes that play important roles in the development of atherosclerosis. We carried out microarray analysis to identify genes differentially expressed between IMA and LAD. Twenty-nine genes showed significant differences in their expression levels between IMA and LAD, which included the *TES* gene encoding Testin. The role of *TES* in the cardiovascular system is unknown. Here we show that *TES* is involved in endothelial cell (EC) functions relevant to atherosclerosis. Western blot analysis showed higher *TES* expression in IMA than in LAD. Reverse transcription polymerase chain reaction and western blot analyses showed that *TES* was consistently and markedly down-regulated by more than 6-fold at both mRNA and protein levels in patients with CAD compared with controls without CAD ($P = 0.000049$). The data suggest that reduced *TES* expression is associated with the development of CAD. Knockdown of *TES* expression by small-interfering RNA promoted oxidized-LDL-mediated monocyte adhesion to ECs, EC migration and the transendothelial migration of monocytes, while the over-expression of *TES* in ECs blunted these processes. These results demonstrate association between reduced *TES* expression and CAD, establish a novel role for *TES* in EC functions and raise the possibility that reduced *TES* expression increases susceptibility to the development of CAD.

INTRODUCTION

Atherosclerosis is the leading cause of death and disability for both men and women in the world (1). A common treatment for coronary artery disease (CAD) is coronary artery bypass grafting (CABG) surgery. While several arteries and veins can be used as blood conduits in this procedure, internal mammary artery (IMA) grafts have demonstrated significantly better long-term results with lower complications irrespective of age, gender, race or left ventricular function (2,3). For example, the patency of IMA grafts 7 to 10 years after CABG is 85–95% when compared with saphenous vein

grafts, which readily re-occlude with atherosclerosis, hypertrophies or become fibrotic (4). Furthermore, the IMA patency 20 years after surgery is even >90% when the graft serves the left anterior descending (LAD) coronary artery territory (4). Interestingly, the only disease-prone site of an IMA graft is at the anastomotic site grafted to the coronary artery, suggesting surgical trauma as the inciting factor (5). Recommendations are that its use for CABG is preferred in all situations (6,7).

The proximal LAD is the coronary artery to which the IMA is most frequently grafted with resultant improved long-term survival (7). A high-grade proximal LAD stenosis is a major risk factor for sudden death. An acute coronary occlusion in LAD is more likely to cause a fatal myocardial infarction (MI) compared with all the other coronary arteries with the exception of the left main trunk (4). IMA are comparable in size to the proximal LAD, and both arteries are arteries subject to the same pro-atherogenic environment; yet IMA are atherosclerotic-resistant and the proximal LAD is athero-prone (7). The contrasting properties of these two arteries are an interesting subject to identify the molecular mechanisms of this differential propensity to develop and not to develop disease.

We hypothesize that gene expression differences are the key to distinguish IMA from the proximal LAD with respect to their sensitivities to the development of atherosclerosis. To test this hypothesis, we used oligonucleotide microarrays to identify the genes that are differentially expressed in IMA compared with LAD. We found 29 genes whose expression levels were statistically different. The functions of these genes provide insights as to why IMA are resistant to the progression of atherosclerosis and may aid in the development of therapeutic options to prevent such life-threatening disease in the coronary artery. One of these 29 genes, namely the *TES* gene, was selected for follow-up functional studies for its effects on endothelial cell (EC) functions relevant to the pathogenesis of CAD.

The human *TES* gene (protein product is Testin) was originally identified in 1995 and was noted to have its highest expression in the testes, yet it is widely expressed in normal tissues. It is located in the human FRG7G region on chromosome 7q31.2, and encodes a 421-amino acid protein that contains a Prickle, Espinas and Testin domain at the N-terminus (amino acids 92–195) and three LIM domains at the C-terminus (LIM1 amino acid, 236–296, LIM2 301–357, LIM3 361–414).

Testin localizes in the cytoplasm and is a component of focal adhesions and cell-to-cell junctions. The *TES* gene was proposed to be a tumor suppressor gene based on the finding that when challenged with a carcinogen, *TES* knockout mice showed an increased susceptibility to tumor formation (8). However, the functional role of *TES* in the cardiovascular system is previously unknown. In this study, we identified *TES* as a novel gene which is highly expressed in the endothelium of coronary arteries and involved in EC functions relevant to atherosclerosis. We found that the expression of *TES* was significantly higher in IMA than LAD, and its expression was lower in LAD tissues from CAD patients than from nondiseased coronary arteries. Functional studies were carried out to determine the effects of *TES* on cellular properties relevant to atherosclerosis, including monocyte adhesion to ECs, oxidized-LDL (oxLDL)-induced transendothelial migration of monocytes and EC migration.

RESULTS

Microarray analysis identified 13 genes showing increased expression levels and 16 genes showing reduced expression levels in IMA compared with LAD

One objective of this study was to perform global microarray analysis to compare IMA to proximal LAD and to identify the genes differentially expressed between the two groups of tissue samples. This will give a better understanding to the contrasting tendencies of these two arteries to develop atherosclerosis from a transcriptome perspective. The microarray analysis involved 11 study subjects ($n = 5$ and 6 for IMA and LAD, respectively) (Supplementary Material, Table S2). We used the U95A set of microarrays (Affymetrix) and carried out gene expression analysis with GeneSpring Microarray Analysis Software. The analysis generated a final list of 29 genes showing $\geq$3-fold differential expression between IMA and LAD (Supplementary Material, Table S1). Twelve genes including *ATF3*, *TINUR*, *LMNA*, *HSP70*, *TES* and *RGS5* showed increased expression in IMA compared with LAD. In contrast, expression of 17 genes including *CALB2*, *RASF-A-PLA2* and *PECAM1* were lower in IMA than in LAD (Supplementary Material, Table S3).

To confirm the microarray results, we randomly selected 7 of the 29 genes, including *HSP70*, *HBGR2*, *TINUR*, *CD163 Antigen*, *CDH7*, *AQP7* and *F13A1,* for real-time reverse transcription polymerase chain reaction (RT–PCR) analysis with the RNA samples used for the original microarray analysis. The results from the confirmation study are in general agreement with the microarray analysis with regard to the median fold changes (Supplementary Material, Table S3).

Expression of a subset of 11 genes in IMA and LAD arterial samples from an independent cohort of 30 human subjects

Among the 29 genes identified by the microarray analysis (Supplementary Material, Table S1), we selected 11 genes for further validation studies (Table 1). This set of genes was selected based on mostly their physiological relevance to or potential functions related to atherosclerosis. Real-time RT–PCR analysis was carried out for each gene using matched IMA and LAD samples simultaneously harvested from 30 independent subjects. The *RGS5*, *LMNA*, *TES* and *NR4A1* genes showed 2-fold or more expression in IMA than in LAD (Table 1). *F13A1*, *RASF-A-PLA1* and *PECAM1* showed more than 2-fold less expression in IMA compared with LAD (Table 1). A <2-fold difference was found for *ATF3*, *TINUR*, *HSP70* and *CD163* for their expression between IMA and LAD (Table 1).

Expression of *TES* is increased in IMA in comparison to LAD

As shown in Table 1, only two genes showed an expression difference of 4-fold or more between IMA and LAD, i.e. *LMNA* and *TES*. *LAMN* mutations were associated with progeria and premature atherosclerosis, but *TES* stood out to be a novel gene that was not previously related to atherosclerosis. Furthermore, *TES* is a component of cell junctional

Table 1. Assessment of candidate genes selected from microarray analysis for their differential expression between the matched IMA and LAD samples using real-time RT–PCR analysis with an independent replication cohort ($n = 30$)

Gene name	GeneBank	Median fold-change on microarray	Median fold-change from replication cohort (real-time RT–PCR)	P-value from RT–PCR
ATF3	L19871	↑8.3	↑1.6	5.6×10^{-6}
TINUR	S77154	↑6.5	↑1.3	2.7×10^{-6}
HSP70	M11717	↑6.1	↑1.8	3.2×10^{-8}
RGS5	AB008109	↑5.9	↑2.8	4.9×10^{-8}
LMNA	M13452	↑5.0	↑4.5	1.52×10^{-6}
Testin2	AL050162	↑4.3	↑4.0	5.5×10^{-9}
NR4A1	L13740	↑3.7	↑2.0	4.6×10^{-6}
CD163 Antigen	Z22971	↓5.5	↓1.2	4.4×10^{-14}
F13A1	M14539	↓5.5	↓2.2	4.4×10^{-12}
RASF-A-PLA1	M22430	↓3.7	↓3.5	4.8×10^{-11}
PECAM1	A034397	↓3.0	↓2.2	2.2×10^{-9}

complexes, which are critical to the integrity of the EC layer and may seal the paracellular space between ECs (9,10). Thus, *TES* may be involved in the transmigration of monocytes across the EC layer, a main process in the genesis of atherosclerosis. Thus, *TES* was selected for further functional characterization for its relevance to atherosclerosis.

To confirm the real-time RT–PCR results showing increased *TES* expression in IMA compared with LAD, western blot analysis was performed with protein extracts from the IMA and proximal LAD tissues. The analysis showed significant up-regulated expression of Testin in IMA compared with LAD ($P = 0.014$) (Fig. 1A).

Because *TES*/Testin expression is increased in response to testosterone in Sertoli cells (11), it is interesting to determine whether the same occurs in arterial ECs. We separated the 30 study subjects into the male group ($n = 26$) and the female group ($n = 6$) and analyzed the expression level of the Testin protein in each group. As shown in Supplementary Material, Figure S1, western blot analysis showed that the expression of Testin was significantly up-regulated in IMA compared with LAD in both males and females. However, Testin expression did not show any difference between males and females in any one specific artery, either LAD or IMA (Supplementary Material, Fig. S1), suggesting that the Testin expression level in arteries is independent of gender.

Predominant expression of the testin protein in the endothelium layer of arteries

To determine the expression profile of the Testin protein in arteries, we carried out immunostaining studies of IMA and LAD tissue sections with an anti-Testin antibody. The immunostaining signal for Testin co-localized with CD31 in both IMA and LAD, indicating strong *TES* expression in arterial ECs, i.e. the endothelium (Fig. 1B). Because Testin was expressed predominantly in the endothelium of arteries,

follow-up functional studies of *TES* and Testin focused on ECs from these two arteries.

TES/Testin expression in LAD is decreased in CAD patients compared with non-CAD subjects

Because the expression level of *TES*/Testin is higher in atherosclerotic-resistant IMA than in atherosclerotic-prone LAD, we hypothesized that *TES* is involved in contributing a resistance to atherosclerosis. To test this hypothesis, we assessed the expression levels of the *TES* gene in coronary arteries from 10 CAD patients in comparison to 10 non-diseased coronary arteries by real-time RT–PCR analysis. Expression of *TES* was markedly reduced by 6-fold in CAD patients compared with non-CAD subjects ($P = 0.000049$) (Fig. 2A).

Western blot analysis of protein extracts from LAD with a different cohort of 10 CAD patients and 10 non-CAD controls further showed that Testin expression was decreased in diseased coronaries in comparison with disease-free arteries (Fig. 2B and C) ($P = 0.00023$). These results suggest that reduced *TES* expression is associated with the development of CAD. When we divided the patients by gender ($n = 8$ males and 2 females), there was no difference in the expression of Testin between males and females ($P > 0.05$), confirming the previous conclusion that the Testin expression level is independent of gender in coronary arteries.

TES affects oxLDL-mediated monocyte adhesion to LAD ECs

To identify a cellular mechanism for the association between *TES*/Testin expression and its sensitivity to atherosclerosis, we characterized the effects of over-expression of *TES* in LAD ECs by transient transfection with an expression construct for 6xHis-tagged TES (empty vector as the negative control) or knock-down of *TES* expression by RNA interference (scrambler as control) with two different small-interfering RNAs (siRNAs) (Fig. 3). Western blot analysis showed successful over-expression of Testin in LAD ECs by ∼3-fold with transient transfection of the *TES* expression plasmid compared with the vector, and the two different *TES* siRNAs effectively reduced the expression level of Testin in LAD ECs by 75% compared with the scramblers (Fig. 3).

Because adhesion of blood monocytes to the ECs of coronary arteries is a significant step in initiating atherosclerosis, we first assessed the effect of over-expression of *TES* and knock-down of *TES* expression in LAD ECs on monocyte adhesion. As shown in Figure 4, treatment of ECs with oxidized low density lipoprotein (oxLDL) (versus native un-oxLDL) induced the expression of cell adhesion molecules reflected by the increased adhesion of monocytes to the ECs in all the groups, yet the over-expression of *TES* decreased the adhesion of monocytes to ECs (compare *TES* with vector, $P = 0.022$ in the upper panel; and $P = 0.00347$ in the lower panel). At the same time, knockdown of *TES* expression increased adhesion of monocytes to ECs, (compare siRNA1 with scrambler, $P = 0.021$ in the upper panel and siRNA2 to scrambler $P = 0.007391$ in the lower panel) (Fig. 4). These results indicate that *TES* plays an important role in the adhesion of monocytes to ECs, an event related to the genesis of atherosclerosis.
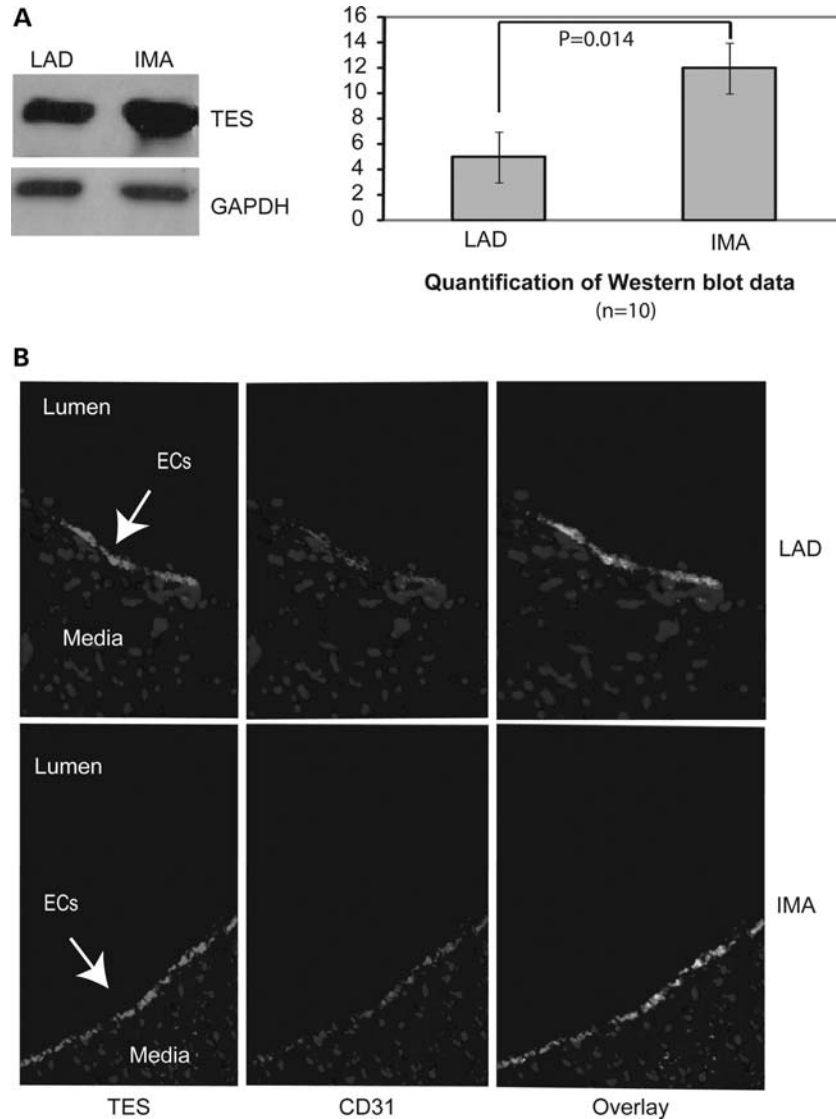
Figure 1. Increased *TES* expression in IMA compared with LAD, and strong expression of Testin in ECs. (**A**) Western blot analysis with protein extracts from IMA and LAD (left panel). Western blot images were scanned, quantified and plotted ($n = 10$, right panel). *TES* expression was higher in the atherosclerotic-resistant IMA than in atherosclerotic-prone LAD ($n = 10$/group) ($P = 0.014$). (**B**) Immunostaining analysis of IMA and LAD with an anti-Testin antibody. The tissues were co-immunostained with an anti-CD31 antibody, a marker for ECs. The signal for Testin co-localized with the CD31 signal, indicating that Testin is expressed strongly in ECs.

TES is involved in transmigration of monocytes across oxLDL-treated LAD ECs

Because Testin is a component of cell-to-cell junctions (9), we hypothesized that Testin plays a role in the transendothelial migration of monocytes, which is a critical process in development of CAD. *TES* was over-expressed in ECs derived from the LAD coronary artery, and these ECs were exposed to oxLDL (0.25 mcg/ml for 24 h). The effect of *TES* overexpression in ECs on the transmigration of HL-60 monocytes was measured after 24 h by plating monocytes onto confluent ECs adherent to the bottom of the Boyden chamber (Fig. 5). Overexpression of *TES* in ECs significantly reduced transmigration of monocytes across the EC layer (Fig. 5, compare *TES* with vector, $P = 0.00000000051$ in the upper panel and $P = 0.00417$ in the lower panel). Knockdown of *TES*

expression by *TES*-specific siRNA increased transmigration of monocytes across the EC layer (Fig. 5, compare siRNA1 with scrambler, $P = 0.028$ in the upper panel and siRNA2 to scrambler, $P = 0.000113$ in the lower panel) (Fig. 5). The results indicate that *TES*/Testin plays a significant role in affecting the migration of monocytes across the EC layer, a cellular process critical to the development of atherosclerosis.

TES is involved in migration of LAD ECs, but did not play a role in EC apoptosis

The effects of *TES* on other properties of ECs, including migration and apoptosis, were also analyzed. For EC migration, we analyzed the effect of over-expression of *TES* and knockdown of *TES* using a scratch assay. Over-expression of *TES*
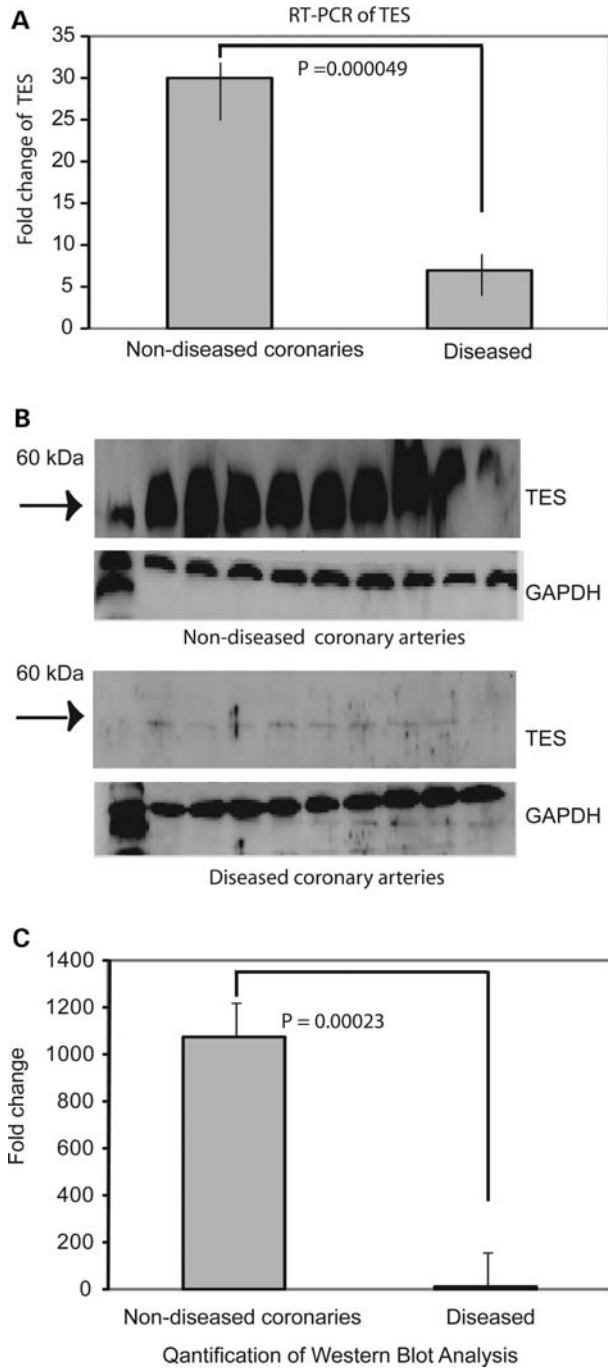
Figure 2. Decreased *TES*/Testin expression in coronary arteries from CAD patients compared with non-CAD samples. (**A**) *TES* mRNA expression from 10 non-diseased coronaries compared with 10 diseased coronary arteries by real-time RT–PCR. The mRNA expression level of *TES* in patients with CAD is significantly lower than in non-CAD individuals. (**B**) Testin protein expression by western blot analysis with an additional cohort of 10 CAD patients and 10 non-CAD individuals shows that Testin expression is lower in patients with CAD. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as loading control. (**C**) Western blot images in (B) was scanned, quantified and plotted (*n* = 10).

significantly inhibited the migration of ECs (Fig. 6, compare *TES* with vector; $P = 0.000000079$ in the upper panel; $P = 0.0037$ in the lower panel). Knockdown of *TES* expression
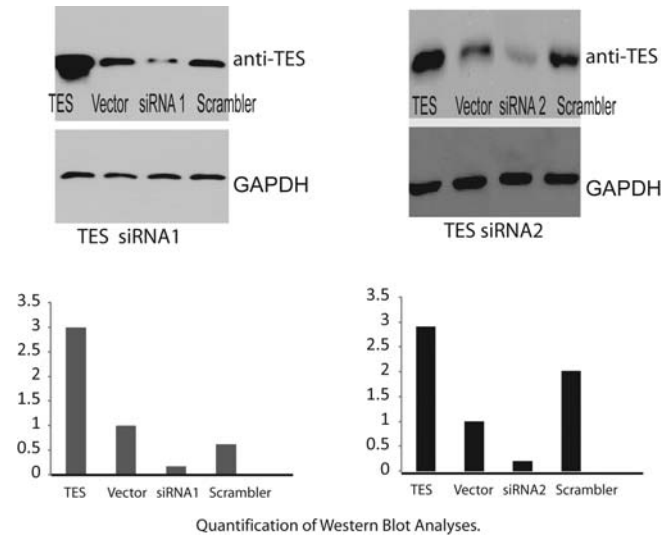

Figure 3. Overexpression and knockdown of *TES*/Testin expression. Western blot analysis shows that Testin was successfully overexpressed in LAD-derived ECs by transient transfection (empty vector as control) and that Testin expression was successfully knocked down by *TES* siRNA1 and siRNA2 (scrambler as control). GAPDH served as loading control.

increased migration of ECs (Fig. 6, compare siRNA1 with scrambler, $P = 0.006$ in the upper panel; siRNA2 to scrambler, $P = 0.003$ in the lower panel) (Fig. 6). These results suggest that *TES* plays an important role in the migration of ECs.

For EC apoptosis, we transfected coronary ECs with a *TES* expression plasmid to over-express this gene and then analyzed for apoptosis using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The over-expression of the *TES* gene did not induce apoptosis in ECs (data not shown).

DISCUSSION

This study was designed to identify novel genes that confer a resistance to or protection from atherosclerosis by investigating the fundamentally different gene expression patterns between atherosclerotic-resistant IMA and atherosclerotic-prone proximal LAD in human subjects. The study successfully identified a novel gene, *TES* (protein product Testin), whose expression level was associated with atherosclerosis. The expression level of *TES*/Testin was higher in IMA compared with LAD at both the mRNA and protein levels. Similarly, the expression level of *TES*/Testin was significantly higher in non-diseased coronary arteries than that in diseased arteries. Therefore, we conclude that decreased *TES*/Testin expression is associated with the development of CAD.

We then carried out functional studies to identify the cellular/molecular mechanisms by which *TES* affects the function of ECs that are relevant to the development of atherosclerosis. The pathogenesis of atherosclerosis starts with the adhesion of monocytes to ECs, followed by their transendothelial migration into the intima of LAD. The monocytes differentiate into macrophages which release cytokines, amplifying the inflammatory process and culminating in foam cells, the hallmark of atherosclerosis (10). Our functional studies with the
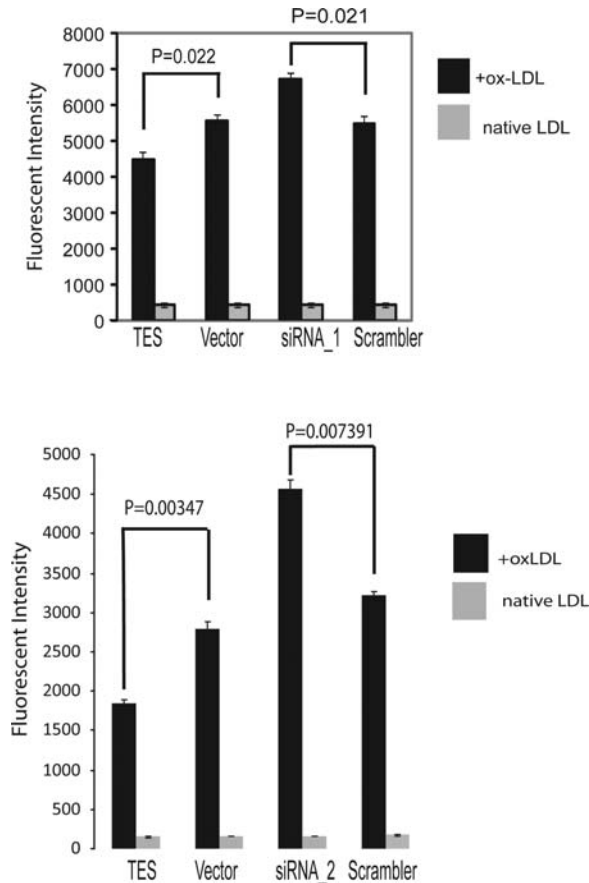
Figure 4. Effects of *TES*/Testin on monocyte adhesion to LAD ECs. The treatment of ECs with oxLDL dramatically increased their adhesion to monocytes. Over-expression of Testin (compare TES with vector) in ECs appeared to decrease adhesion of ECs to monocytes. Knockdown of Testin expression in ECs increased adhesion of ECs to monocytes (compare siRNA1 or siRNA2 with scrambler).

Figure 5. Transendothelial monocyte migration assays using LAD ECs with overexpression of *TES* or knockdown of *TES* expression. The overexpression of Testin in ECs significantly reduced transmigration of monocytes across the EC layer (compare TES with vector). Knockdown of Testin by siRNA1 or siRNA2 showed more transmigration of monocytes across the EC layer (compare siRNA1 or siRNA2 with scrambler).

overexpression of *TES* or the knockdown expression of *TES* suggest that it plays an important role in monocyte adhesion to the endothelium and the transendothelial migration of monocytes. We showed that overexpression of *TES* decreased monocyte adhesion to ECs and migration of monocytes across the EC layer, while knockdown of *TES* increased these functions. The molecular mechanism by which Testin affects monocyte adhesion to ECs is unknown, but future identification of specific adhesion molecules that may be affected by overexpression or knockdown of *TES* expression may provide important insights. The function of *TES* in the coronary artery may parallel its function in the testes where it serves a role as being part of the tight junction between adjacent, germinal epithelial Sertoli cells (9,11,12). In the testes, Testin contributes to the blood-testes barrier to protect the developing germ cells from the systemic circulation and blunts the diffusion of proteins that can damage the developing gametes. Correspondingly, Testin may strengthen the endothelium to retard the binding to and passage of monocytes through the interjunctional space between ECs into the intima, the initial event leading to atherosclerosis.

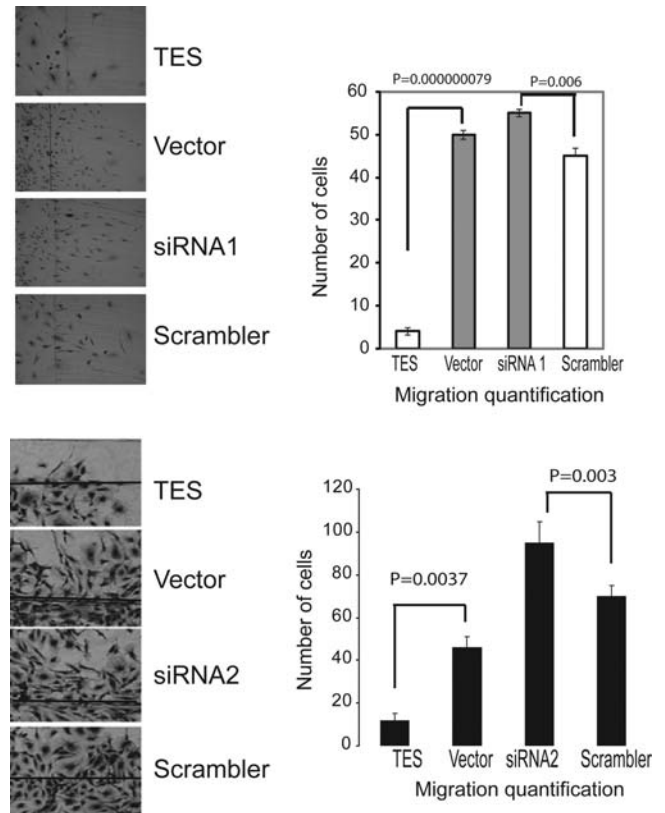We also found that overexpression of *TES* inhibited EC migration, whereas knockdown of *TES* expression increased EC migration (Fig. 6). Testin may regulate EC migration through a Mammalian enabled (Mena) dependent mechanism (13). Testin may displace Mena from focal adhesion protein complexes at the leading edge of a migrating cell, disrupting the protein complexes critical for cell migration (13) and inhibiting EC migration. The relevance of Testin-mediated inhibition of EC migration to atherosclerosis is not clear. In fact, the role of EC migration in atherosclerosis is unknown. We speculate that EC migration may be detrimental to atherosclerosis by creating gaps between ECs, whereas these gaps promote monocyte transmigration across the endothelium and development of atherosclerosis. Overexpression of Testin may inhibit EC migration, strengthen the integrity of the endothelium, decrease transendothelial migration of monocytes and contribute a resistance to atherosclerosis.

A previous study by Qin *et al*. (14) used suppression subtractive hybridization (SSH) with three pooled porcine mRNA samples to identify the gene expression differences in IMA and LAD denuded of ECs. Qin *et al*. identified 24 genes using porcine tissues, but none of these genes were found in common with our final list of genes expressed in humans, and none of their genes were characterized by follow-up functional studies. Qin *et al*. (14) focused on vascular smooth muscle cells only, and this may explain why the genes identified by the two studies are different. Furthermore,
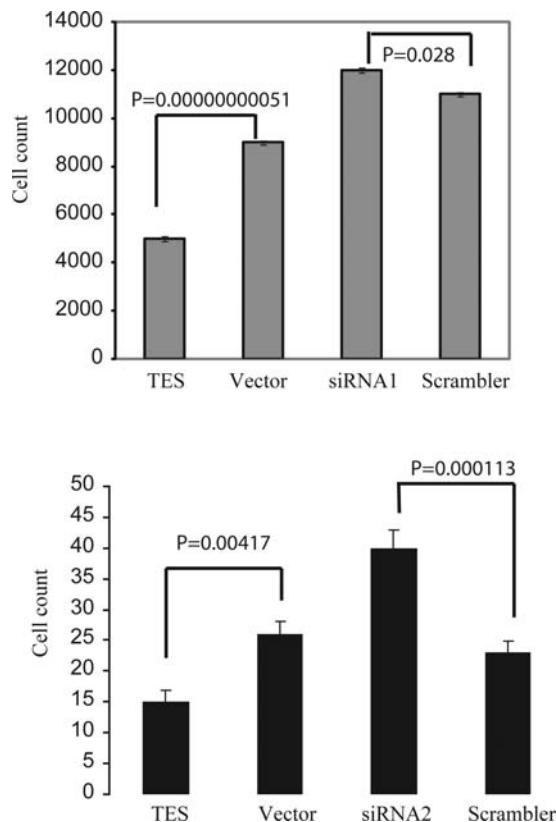
Figure 6. Effect of *TES*/Testin on EC migration. Over-expression of Testin in ECs significantly inhibited the migration of ECs (compare TES with vector). Knockdown of Testin increased EC migration (compare siRNA1 or siRNA2 with scrambler).

the SSH strategy is limited by the number of genes to be analyzed or cDNA clones selected for sequencing. In contrast, our study is the first global gene expression analysis between IMA and LAD using human samples and our microarray analysis is more a global strategy that analyzes more than 12 000 genes simultaneously.

We found that the expression level of *TES*/Testin was significantly reduced in CAD tissue samples when compared with non-CAD samples, but the underlying mechanism is not clear. There are several possibilities. First, transcriptional silencing resulting from CpG island methylation at the 5′ end of *TES* was well-documented in ovarian carcinomas, gastric cancer and childhood acute lymphoblastic leukemia (8). Thus, epigenetic effects of atherosclerosis in the 5′-promoter and regulatory region of *TES* may reduce the expression level of *TES* in CAD tissues. Secondly, the cellular composition of the vessels in CAD patients versus controls may also be a potential cause for reduced *TES* expression in CAD tissues. In this case, reduced *TES* expression is predicted to exacerbate the development of atherosclerosis because the functional impact of increased monocyte adhesion to ECs and increased transendothelial migration of monocytes caused by decreased *TES* expression is considered to be a risk factor for the development of atherosclerosis. Thirdly, the expression level of *TES* may be sensitive to inflammation, a key process during atherosclerosis, although *TES* was not

found in the list of genes whose expression in primary human ECs was affected by treatment with oxidized palmitoyl arachidonyl phosphatidylcholine (15). Finally, the expression level of *TES* may be sensitive to regulation by single nucleotide polymorphisms (SNPs). We found that *TES* expression is associated with 753 expression quantitative trait loci (eQTLs). Only one is a cis-eQTL consisting of 30 SNPs (rs2401967, rs10249355, rs12539389, rs1917058, rs1464775, rs7780647, rs10239615, rs4727826, rs11770268, rs10953810, rs12668723, rs10245914, rs11773466, rs1004108, rs1004109, rs1962150, rs6466570, rs1048508, rs3807979, rs2402060, rs12672890, rs10242959, rs12673920, rs10266135, rs10269717, rs10239692, rs10953816, rs10953817, rs11761066, rs1528243). The cis-eQTL (physical location, 114,773,053–116,470,664) spans the entire *TES* gene (physical location, 115,850,547–115,898,837). Two separate trans-eQTLs for *TES* contain SNP rs12510359 in the *PALLD* gene on chromosome 4 and rs137625 in the *TAS2R50* gene on chromosome 12, which associated with MI in a study by Shiffman *et al*. (16). Genome-wide association studies have identified >30 susceptibility variants and/or loci, but none of them covers the *TES* gene. We have performed a small-scale case control association study (768 MI patients and 768 controls) with two tagSNPs that capture the information of common variants in TES (a minor allele frequency of >35%). No significant association was detected, but it remains to be determined whether rare variants in *TES* are association with CAD and MI.

There is a limitation of our study. This project was initiated and prompted by the tissue samples that had already been available from the Cleveland Clinic Heart Transplantation program, thus our initial cohort of LAD samples for expression profiling came from heart transplants at Cleveland Clinic. Because IMA were not available from the same heart transplantation patients, we matched the LAD samples with the IMA samples from autopsy for age and gender and past medical history. From the standpoint of statistics, this study design is superior because the two groups of samples are independent and random. However, the increased sample heterogeneity may increase type 1 errors. To minimize this complication, we did replicate our genechip findings from this initial cohort in yet a much large patient population ($n = 30$) to validate these initial findings. Despite the limitation of tissue heterogeneity, which is unavoidable with human tissue studies, our study identified at least one new gene, *TES*, that significantly affects EC functions and is associated with CAD because a decreased expression level of *TES* correlates with the development of CAD. Thus, *TES* may play an important role in cellular processes relevant to atherosclerosis. Future studies with $TES^{-/-}$ knockout mice under a background of $ApoE^{-/-}$ or $LDLR^{-/-}$ are needed to unequivocally demonstrate whether *TES* is directly involved in resistance to atherosclerosis *in vivo*.

In conclusion, we performed the first global gene expression profiling study on the human atherosclerotic-resistant IMA when compared with atherosclerotic-prone proximal LAD. This study identified a novel gene, *TES*, which is associated with CAD. We showed that *TES* expression was significantly decreased in LAD tissues from patients with CAD compared with non-CAD patients. Functional studies showed that *TES*

plays important roles in monocyte adhesion to ECs and trans-endothelial migration of monocytes which are critical to the initiation of atherosclerosis. Continued future functional characterization of *TES* may define it as a novel target for developing therapeutic strategies to treat CAD.

MATERIALS AND METHODS

Tissue samples of human arteries

Arteries were obtained from explanted hearts through the Cleveland Clinic Heart Transplant Program, and unmatched or rejected healthy donor hearts from LifeBanc of Northeast Ohio and the Cuyahoga County Coroner's office. Each LAD was carefully and thoroughly examined for the presence or absence of atherosclerosis by microscopic examinations of consecutively dissected serial segments of the entire artery by cardiovascular pathologists. We selected a segment of the proximal LAD for this study. Informed consent was obtained from the participants (or their families at post-mortem exams) according to the standards established by the Cleveland Clinic Foundation Institutional Review Board on Human Subjects and the Cuyahoga County Coroner. Arteries were cleaned of adjacent adipose and myocardial tissue in explanted hearts or from cadavers (17). The adventitial layer remained intact. The specimens were snap-frozen in liquid nitrogen and stored at $-80°C$ as previously described until used (18).

The initial cohort of human coronary arteries for expression profiling was harvested from hearts with dilated cardiomyopathies (prior heart catherizations documenting no atherosclerosis, $n = 4$) and rejected normal heart donors ($n = 2$) (Supplementary Material, Table S2). The average age was 52. The coronaries were matched with the IMA. For follow-up RT–PCR and western blot analyses, we compared the arterial gene expression between these two arteries harvested from the same patient but from a larger group ($n = 30$). The average age was 54.

Isolation of RNA and oligonucleotide arrays

Total RNA was isolated from the arteries using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and used for RT–PCR analysis or microarray studies. Microarray analysis was performed as previously described by us (17). The CWRU-CCF Gene Expression Core converted total RNA to cRNA labeled by biotinylation. The biotinylated cRNA probe was hybridized to a test chip containing only a few commonly expressed genes, such as actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), to establish the quality of the probe. If the test hybridization was successful, then the microchips of interest were probed under the same conditions. Between the two groups (IMA versus LAD), there were not any statistically significant differences ($P > 0.05$) in the percentage of genes expressed, Q-scores or background noise on the microarrays.

The Human Genome U95A Array (Affymetrix) was used. Each array contains $\sim 12\,000$ unique genes. GeneSpring (Agilent technologies) was used for data mining as previously described (19). We used the standardized statistical algorithm

as followed on the website (www.agilent.com). A cohort of six proximal LAD coronary arteries and five left IMA were used and treated as two groups of replicates. Before any analyses were performed, all data were screened for any genes with a negative average difference value (and negative values set to zero). The average difference after log-transformation between LAD and IMA was compared by using the Welch analysis of variance test, and genes showing $P < 0.001$ were selected for further analysis. This initial analysis generated a list of 2219 genes. Then, we filtered the gene list by accepting genes that were flagged as 'present' on all 11 chips and eliminated them if flagged as 'marginally present or absent'. This generated a list of 53 genes. Finally, we filtered the list with a set difference of 3-fold to identify the genes with large expression differences between the two arteries.

Real-time RT–PCR analysis

Results from the microarray analysis were validated for selected genes by real-time RT–PCR with SYBR Green (VWR). Specific primer pairs were designed for each selected gene (Integrated Biotechnologies, Inc.) (Supplementary Material, Table S4). Relative expression values were calculated as previously described (19,20). The expression level of β_2-microglobulin was used as an internal control. A paired Student's *t*-test was used to compute the *P*-values for expression differences for each gene between the two arteries.

Cell culture and transfection

The IMA primary ECs were purchased from Cell Applications. They came from a single donor, 50-year-old Caucasian male cryopreserved at the second passage in EC basal medium containing 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide. The primary human coronary artery ECs came from a 21-year-old Caucasian male also cryopreserved at the second passage (Cell Applications). Both primary cell lines were characterized by Factor VIII-related antigen expression, DiI-Ac-LDL update and tested negative for several potential biological contaminants.

ECs were transfected with the *TES* expression plasmid using Amaxa (Program U-001) using the HUVEC Nucleofactor Kit (Cat No. VPB-1002). The human *TES* gene was PCR amplified with primers containing in-frame restrictions sites *Eco*RI (GCCAAT TCC TAA GAC ATC CTC TTC TT) and *Bam*HI (AAG GAT CCA GAT GGA GCT GGA AAA). The PCR fragment was digested with *Eco*RI and *Bam*HI and subcloned into pcDNA3.1A (5.5 kb 6X histidine-tagged vector) (Invitrogen). The identity of the construct was verified by direct DNA sequence analysis of the entire insert using the BigDye Terminator Cyclic Sequencing Ready Reaction Kit (Applied Biosystems) using a 3100 Genetic Analyzer (Applied Biosystems). Cells were used for assays 24 and 48 h after transfection.

siRNA for *TES* was purchased from Integrated DNA technologies: siRNA1, sense—rGrCrA rGrCrA rCrCrU rGrCrC rArUrG rArArC rUrCrC rUrGG T and antisense—rArCrC rArGrG rArGrU rUrCrA rUrGrG rCrArG rGrUrG rCrUrG rCrArG; siRNA2, sense—rGrGrGrCrUrUrArGrGrUrCrArCr GrArGrCrArA and antisense–rUrUrGrCrurCrGrUrGrArCr

CrUrArArGrCrCrC. The optimal dose to interfere with *TES* mRNA expression was empirically determined to be 1 μM.

Preparation of protein extracts by the delipidation method

The IMA tissues or proximal LAD coronary arteries (0.2 g/sample) were lysed in a homogenization buffer for 15 min at 35°C using the delipidation method as previously described (20). Protein extracts from IMA or LAD ECs grown *in vitro* were prepared with M-PER Mammalian Protein Extraction Reagent (Pierce) and quantified with BCA Protein Assay Kit (Pierce).

Western blot analysis

Western blotting was performed as previously described (20). The primary antibodies used included the antiserum for CD31 (PharMinger) and for GAPDH (Santa Cruz). The antibody against Testin was kindly provided by Dr Yingli Zhong. Quantification of bands was made with Melanie 2D gel software analysis (SIB).

Immunostaining

Cryo-sections (6 μm) of the proximal LAD and IMA were fixed with paraformaldehyde (4%) and permeablized with Triton X-100 (0.2%) as previously described (19) and incubated with an anti-Testin antibody. Anti-rabbit IgG conjugated with fluorescein isothiocyanate (Sigma) was used to visualize TES. The sections were counterstained with 4′,6′ diamino-2-phenylindole, 2HCl for staining nuclei (Vectashield with OAD, Vector Labs). The immunostaining signals were visualized with a Zeiss Axiophot fluorescence microscope and the images were captured with Imageplus 6.0.

Monocyte adhesion assays

Fully confluent ECs were grown in CoStar96 well plates after being seeded at a density of 5000 cells/well and stimulated with oxLDL (Sigma) (0.25 mg/ml) for 6 h. Human HL-60 promyelocytic leukemia cells were cultured in Iscove's modified Dulbecco's media, fluorescently labeled with Calcein AM (Invitrogen) for 60 min at 37°C, and seeded into each well coated with ECs at a density of 3×10^5 per well. After 1 h, the media were aspirated and wells were washed gently with PBS three times. Fluorescent intensity was measured in each of the 96 wells with a standard fluorometer with the CytoFluoro-II program (Applied Biosystems) (exit: 485/20, emission 530/30, gain of 65) to determine the fluorescent units in each well. The rational for using HL-60 cells is that they represent immature monocytes *in vivo* which attach to and transmigrate through ECs and then may differentiate into different mature lineages.

Cell migration assays

ECs were transfected as mentioned above and plated at a density of 0.5×10^6 cells per cm^2 in 35 mm six-well plates. The media were changed 24 h later. Then, the confluent monolayers were wounded with a single edge razor and cells on the right side of the wound were removed. After 48 h, the cells were stained with Coomassie Brilliant Blue for 10 min followed by Glacial acetic acid/methanol destaining buffer, and photographed with Image-Pro Plus and analyzer 7.0 (Mediacy-bernetics). The number of cells which migrated passed the wound were counted under a 4× power field.

Transmigration of monocytes assays

HL-60 cells (3×10^5) were pipetted into 0.4 μm pore chambers (Corning) within a Boyden chamber, which had *TES* transfected ECs plated 24 h prior and then exposed to 0.25 μg of oxLDL. FBS was used as a chemo-attractant and diluted in serum-containing Dulbecco's modification of Eagle's medium. The HL-60 cells were collected and counted from the bottom chamber 48 h after their administration with a Bright-Line Hemacytometer (Hausser Scientific) using an Olympus CKX 41 microscope. Each experiment was repeated in triplicate.

Apoptosis assays

Coronary artery ECs were transfected with a *TES* expression plasmid and cultured for 48 h. The ECs were suspended in 1% (w/v) paraformaldehyde in PBS (pH 7.4) at a concentration of $1-2 \times 10^6$ cells/ml. The cells were placed on ice for 30–60 min, centrifuged for 5 min at 300*g* and the supernatant discarded. ECs were studied by the TUNEL apoptosis assay using the APO-BRDU Kit (BD Pharmingen) according to the manufacture's instructions. The cells were resuspended and exposed to 70% (v/v) ice-cold ethanol for 30 min, and sent to the CCF Cytology Core for apoptosis assays.

Statistical analysis

For EC functional assays, the difference between the two groups of variables was compared by the paired Student *t*-test. A *P*-value of <0.05 was considered significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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