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Influence of a Coronary Artery Disease–Associated Genetic Variant on *FURIN* Expression and Effect of Furin on Macrophage Behavior

Guojun Zhao, Wei Yang, Jingchun Wu, Bairu Chen, Xu Yang, Junhui Chen, David G. McVey, Catherine Andreadi, Peng Gong, Tom R. Webb, Nilesh J. Samani, Shu Ye

Objective—Genome-wide association studies have revealed a robust association between genetic variation on chromosome 15q26.1 and coronary artery disease (CAD) susceptibility; however, the underlying biological mechanism is still unknown. The lead CAD-associated genetic variant (rs17514846) at this locus resides in the *FURIN* gene. In advanced atherosclerotic plaques, furin is expressed primarily in macrophages. We investigated whether this CAD-associated variant alters *FURIN* expression and whether furin affects monocyte/macrophage behavior.

Approach and Results—A quantitative reverse transcription polymerase chain reaction analysis showed that leukocytes from individuals carrying the CAD risk allele (A) of rs17514846 had increased *FURIN* expression. A chromatin immunoprecipitation assay revealed higher RNA polymerase II occupancy in the *FURIN* gene in mononuclear cells of individuals carrying this allele. A reporter gene assay in transiently transfected monocytes/macrophages indicated that the CAD risk allele had higher transcriptional activity than the nonrisk allele (C). An analysis of isogenic monocyte cell lines created by CRISPR (clustered regularly interspaced short palindromic repeats)-mediated genome editing showed that isogenic cells with the A/A genotype for rs17514846 had higher *FURIN* expression levels than the isogenic cells with the C/C genotype. An electrophoretic mobility shift assay exhibited preferential binding of a nuclear protein to the risk allele. Studies of monocytes/macrophages with lentivirus-mediated furin overexpression or shRNA (short hairpin RNA)-induced furin knockdown showed that furin overexpression promoted monocyte/macrophage migration, increased proliferation, and reduced apoptosis whereas furin knockdown had the opposite effects.

Conclusions—Our study shows that the CAD-associated genetic variant increases *FURIN* expression and that furin promotes monocyte/macrophage migration and proliferation while inhibiting apoptosis, providing a biological mechanism for the association between variation at the chromosome 15q26.1 locus and CAD risk.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2018;38:1837-1844. DOI: 10.1161/ATVBAHA.118.311030.)**

Key Words: coronary artery disease ■ furin ■ macrophages ■ monocytes ■ polymorphism, single nucleotide

Genome-wide association studies have shown a robust association between genetic variation on chromosome 15q26.1 and coronary artery disease (CAD) susceptibility.[1–4] However, the biological and pathological mechanism underlying this genetic association is still unknown. At this locus, the lead CAD-associated single nucleotide polymorphism (SNP), rs17514846, is located within a noncoding region of the *FURIN* gene. It is plausible that rs17514846 per se, or another SNP in linkage disequilibrium (LD) with rs17514846, has an effect on *FURIN* gene expression. Findings about whether this is the case would be valuable for gaining an understanding of the mechanism through which variation at this locus influences CAD risk.

Furin, encoded by the *FURIN* gene, is an important proprotein convertase responsible for the activation of several growth factors[5–7] and metalloproteinases.[8–12] There is evidence implicating furin in atherosclerosis, the pathology underling CAD. Studies have shown that furin is expressed in atherosclerotic plaques, with substantially higher levels in advanced lesions.[13,14] In advanced atherosclerotic plaques, furin is produced primarily by macrophages.[13,14] However, it is unclear as to whether furin has a functional effect on macrophage behavior, which warrants investigation because macrophages play important roles in atherogenesis.[15–17]

Therefore, we investigated in this study: (1) whether the CAD-associated variant at the 15q26.1 locus has an effect on

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From the Shantou University Medical College, China (G.Z., W.Y., J.W., B.C., X.Y., S.Y.); Department of Histology and Embryology, Guilin Medical University, China (G.Z.); Department of Minimally Invasive Intervention, Beijing University Shenzhen Hospital, China (J.C.); and Department of Cardiovascular Sciences and NIHR Leicester Biomedical Research Centre, University of Leicester, United Kingdom (D.G.M., C.A., P.G., T.R.W., N.J.S., S.Y.).

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Correspondence to Shu Ye, MD, PhD, Shantou University Medical College, 22 Xinling Rd, Shantou 515100, China. E-mail s_ye@stu.edu.cn or sy127@leicester.ac.uk

Nonstandard Abbreviations and Acronyms	
CAD	coronary artery disease
LD	linkage disequilibrium
SNP	single nucleotide polymorphism

FURIN gene expression in monocytes/macrophages, and (2) whether furin affects monocyte/macrophage migration, proliferation, and apoptosis.

Subjects and Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Bioinformatics Analyses

LDlink (https://analysistools.nci.nih.gov/LDlink/?tab=ldproxy) was used to search for SNPs that are in LD with rs17514846. The University of California Santa Cruz Genome Browser (https://genome.ucsc.edu/) and the WashU EpiGenome Browser (http://epgg-test.wustl.edu/browser/) were used to examine whether rs17514846 and the SNPs in high LD with it are located in genomic regions with transcriptional regulatory marks reported by the Encyclopedia of DNA Elements Project and the Roadmap Epigenomics Mapping Consortium. The GTEx (genotype-tissue expression) Portal (https://www.gtexportal.org/home/) was used to search for a possible association between rs17514846 and gene expression levels.

Subjects

We studied a group of individuals (n=171) with normal coronary angiographic finding. The demographic, biochemical, and clinical characteristics of the study subjects are described in Table I in the online-only Data Supplement. The study subjects were genotyped for SNP rs17514846 and the mRNA levels of the *FURIN*, *FES*, *BLM*, *MAN2A2*, and *UNC45A* genes in blood leukocytes determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. The study was approved by the local ethics committee, and all subjects gave written consent.

Determination of Genotypes

Genomic DNA in blood samples of the study subjects was isolated with the use of FlexGen Blood DNA Kits (Mei5Bio) and genotyped for SNP rs17514846 by TaqMan SNP genotyping assay (C_1244341_10, Applied Biosystems).

RNA Extraction and Quantitative RT-PCR

Total RNA in blood leukocytes of the study subjects was extracted with the use of TRIzol reagent and converted to cDNA using PrimeScript RT reagent Kits, followed by real-time PCR of the *FURIN*, *FES*, *BLM*, *MAN2A2*, *UNC45A*, and *18S rRNA* genes, respectively, by TaqMan Gene Expression Assays (Hs00965485_g1, Hs00171375_m1, Hs00172060_m1, Hs00963969_m1, Hs00218751_m1, and Hs99999901_s1, respectively, Applied Biosystems). *FURIN*, *FES*, *BLM*, *MAN2A2*, and *UNC45A* mRNA levels were standardized against the reference gene *18S rRNA* mRNA levels, using the ΔCt method.

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation was performed with the use of EZ-Magna ChIP A-Chromatin Immunoprecipitation Kits (Millipore). Briefly, mononuclear cells in peripheral blood samples were isolated using Dynabeads FlowComp Human CD14 Kits (ThermoFisher Scientific) and cross-linked with 1% formaldehyde for 10 minutes. Thereafter, cells were lysed on ice, and chromatin DNA was sheared by sonication into fragments of between 0.2 and 1.0 kilobases in length. The chromatin DNA-protein fractions were incubated with an anti-RNA polymerase II (Pol II) antibody (ab5131, Abcam) overnight at 4°C, followed by precipitation of the immunocomplexes using protein A beads. DNA in the immunocomplexes was then isolated and subjected to real-time PCR of the *FURIN* gene and the housekeeping gene *GAPDH*. The ΔΔCt method was used to ascertain differences between genotypes in RNA Pol II occupancy in the *FURIN* gene, standardized against the reference gene *GAPDH*.

Transient Transfection and Luciferase Reporter Assay

Two sets of plasmid constructs were generated using the pGL4.10 Vector (Promega) that contained a firefly luciferase reporter gene. One set of these constructs contained an inserted DNA fragment corresponding to the sequence from nucleotide position −4700 to −4760 (relative to the transcription start site) in the *FURIN* gene, with either A or C at the SNP rs17514846 site at position −4729. The other set of constructs contained 3 tandemly repeated copies of the sequence from nucleotide position −4719 to −4739 (relative to the transcription start site) in the *FURIN* gene, with either A or C at the SNP rs17514846 site at position −4729. These constructs were individually mixed with a pRL-TK plasmid (Promega) containing a *Renilla* luciferase gene and used to transfect cultured monocytes/macrophages (RAW264.7). At 48 hours after transfection, cells were lysed and subjected to luciferase activity assay using a Dual-Glo Luciferase Assay System (Promega). The result is expressed as the ratio of firefly luciferase activity over *Renilla* luciferase activity.

CRISPR-Mediated Genome Editing

With the use of CRISPR (clustered regularly interspaced short palindromic repeats) technology, THP-1 monocytes were subjected to genome editing to generate isogenic cell lines with either the C/C or A/A genotype for rs17514846. In brief, 2 gRNAs (guide RNA; 5′-GTCTGTGGGGGTCTCATTTTC-3′, and 5′-GCCCAC ATCCTCTGTTAAATG-3′, respectively) were separately cloned into the gRNA/Cas9 expression vector containing a green fluorescent protein reporter (p-UG-gRNA-EFS-Cas9-T2A-EGFP-WPRE; Figure I in the online-only Data Supplement). In addition, a homology-directed repair donor vector was prepared by inserting 5′ and 3′ homology arms either side of the to-be-edited site (the inserts were generated by PCR amplification using DNA from THP-1 cells which were of the C/C genotype for rs17514846), followed by site-directed mutagenesis to create a vector containing the A allele of rs17514846. A PGK-Neo-PolyA cassette was also inserted in-between the 5′ and 3′ arms in this vector to provide a means for selection of edited THP-1 cells after transduction (Figure I in the online-only Data Supplement). THP-1 cells were transduced with lentivirus particles containing the gRNAs and the A allele donor vectors in a 1:1:1 ratio. The cells were incubated for 96 hours before fluorescence-activated cell sorting by flow cytometry. Successfully transfected, green fluorescent protein–positive cells accounted for 0.65% of the cell population analyzed. Sorted cells were cultured in 96-well plates under G418 selection to select for successfully edited cells. Subsequently, single-cell clones were expanded in 6-well plates. Every 24 hours, the cell suspension was removed from the wells and centrifuged to remove dead cells. The cell pellet was then resuspended in fresh media containing G418 and returned to the incubator. Daily G418 replenishment and dead cell removal were performed for 2 weeks. The selected cells were then screened via PCR and Sanger sequencing to confirm successful editing of rs17514846 from the parental C/C genotype to A/A.

Parental THP-1 cells, isogenic THP-1 cells (containing the G418 cassette) with the C/C genotype, and isogenic THP-1 cells (containing the G418 cassette) with the A/A genotype were subsequently subjected to quantitative RT-PCR assay of the *FURIN* gene and Western blot analysis of the furin protein.

Western Blot Analysis

Parental THP-1 cells, THP-1 isogenic cells (with either the C/C or A/A for rs17514846) generated by CRISPR-mediated genome editing, RAW264.7 cells, RAW264.7 cells transfected with a control

lentivirus vector or a lentivirus to overexpress furin, and RAW264.7 cells transfected with a control shRNA (short hairpin RNA) or a Furin shRNA, respectively, were subjected to Western blot analysis using an anti-furin antibody (ab3467, Abcam) and an anti-GAPDH antibody (Santa Cruz).

Electrophoretic Mobility Shift Assay

Fluorescent FAM (fluorescein)-labeled double-stranded oligonucleotide probes corresponding to the sequence from nucleotide position −4717 to −4741 (relative to the transcription start site) in the *FURIN* gene, with either A or C at the SNP rs17514846 site at position −4729, were individually incubated with or without nuclear protein extracts from monocytes/macrophages (RAW264.7 cells), in the presence or absence of unlabeled A allele oligonucleotide, unlabeled C allele oligonucleotide, and an unlabeled unrelated oligonucleotide, respectively, at 25°C for 20 minutes. The mixes were subjected to 10% nondenaturing polyacrylamide gel electrophoresis, followed by fluorescence detection of the labeled DNA probes.

Furin Overexpression and Knockdown

For furin overexpression, full-length mouse *Furin* cDNA was amplified by PCR and cloned into the Lenti-EFs-mCherry-WPRE vector, and the correct sequence of the *Furin* gene in this construct was verified by sequencing. This construct and the vector (control vector) were used to transfect cultured monocytes/macrophages (RAW264.7) to overexpress furin; efficient furin overexpression was verified by Western blotting (Figure II in the online-only Data Supplement).

For furin knockdown, 3 shRNAs targeting different regions of the *Furin* transcript were constructed. These and a control shRNA were used to transfect cultured monocytes/macrophages (RAW264.7). Efficiency of furin knockdown was determined by Western blotting that showed that one of these *Furin* shRNAs produced efficient furin knockdown (Figure II in the online-only Data Supplement). This *Furin* shRNA was used in subsequent migration, proliferation, and apoptosis assays.

Transwell Migration Assay

Monocytes/macrophages (RAW264.7) with or without furin overexpression by lentivirus or furin knockdown by shRNA were subjected to transwell migration assay using 24-well plates with 8 μm pore polycarbonate membrane inserts (Corning). In brief, 600 μL culture medium with 10% fetal calf serum was placed in each well, and 10^4 cells in 100 μL culture medium with 0.1% fetal calf serum were placed in the insert. After incubation for 24 hours, cells that had migrated to the lower surface of the membrane of the insert were fixed with methanol and stained with crystal violet. Cell numbers were counted by examining 6 fields per membrane under a microscope.

Proliferation Assay

Monocytes/macrophages (RAW264.7) with or without furin overexpression by lentivirus or furin knockdown by shRNA were subjected to proliferation assay with the use of Cell Counting Kit-8 (Enzo Life Sciences). In brief, cells were cultured in 96-well plates, and a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium solution was added into each well at the same time every day for 5 days. Each day, 2 hours after adding Cell Counting Kit-8 reagent, a colorimetric assay was performed using a microplate reader that measured the absorbance at 450 nm.

Apoptosis Analysis

Monocytes/macrophages (RAW264.7) with or without furin overexpression by lentivirus or furin knockdown by shRNA were subjected to apoptosis assay with the use of annexin V-fluorescein isothiocyanate/propidium iodide Apoptosis Detection kits (KeyGen Biotech). In brief, cells were incubated with fluorescein isothiocyanate–labeled probe for detecting annexin V and with propidium iodide for 15 minutes, followed by flow cytometry analysis.

Statistical Analyses

Linear regression analyses were performed to test a difference between genotypes in the expression levels of *FURIN*, *FES*, *BLM*, *MAN2A2*, and *UNC45A*, respectively, in blood leukocytes of the study subjects, in an additive genetic model, with adjustment for age, sex, hypertension, and diabetes mellitus (values of *BLM, MAN2A2*, and *UNC45A* were first log transformed for normalization of distribution, and their transformed values were used in these analyses). Student *t* test was used to test for differences between experimental groups in Pol II occupancy and luciferase level, respectively (luciferase values of p-C*3-luc and p-A*3-luc were first log transformed for normalization of distribution, and their transformed values were used in the *t* test). A 1-way ANOVA with post hoc test was performed to ascertain differences in *FURIN* expression level between parental THP-1 cells, isogenic THP-1 cells with the C/C genotype, and isogenic THP-1 cells with the A/A genotype. Mann-Whitney test was used to ascertain differences between experimental groups in the number of migrated cells, cell proliferation rate, and apoptosis rate, respectively. A $P<0.05$ in a 2-tailed test was considered statistically significant.

Results

Results of Bioinformatics Analyses

The CAD-associated SNP at the 15q26.1 locus, rs17514846, resides in intron 1 of the *FURIN* gene. It is in high LD ($R^2>0.8$) with 8 other SNPs, 3 of which are also located in the *FURIN* gene (rs11372849, rs8039305, and rs6224) and the other 5 situated in the *FES* gene downstream of *FURIN* (rs2071382, rs11539637 rs7183988, rs7177338, and rs1894401).

A literature search showed that rs17514846 is associated with the expression of the *FURIN*, *FES*, and *MAN2A2* genes in monocytes in a study of 432 volunteers of European ancestry.[18] A bioinformatics analysis of data from the Encyclopedia of DNA Elements Project and Roadmap Epigenomics Mapping Consortium showed that rs17514846 resides in a genomic region that has strong transcriptional regulatory marks (H3K27 acetylation and H3K4 methylation) in primary monocytes and leukemic (K562) cells, whereas the 3 *FURIN* gene SNPs (rs11372849, rs8039305, and rs6224) in high LD with rs17514846 are located in regions without such regulatory features (Figures III and IV in the online-only Data Supplement). The other SNPs (rs2071382, rs11539637, rs7183988, rs7177338, and rs1894401) in high LD with rs17514846 are situated in another genomic region that has lower levels of transcriptional regulatory marks; however, they are not located within the *FURIN* gene but reside in the *FES* gene instead (Figures III and IV in the online-only Data Supplement). The *BLM, MAN2A2*, and *UNC45A* genes are also located nearby and downstream of the *FES*.

As the above bioinformatics evidence indicates that rs17514846 resides in an important transcriptional regulatory region, we hypothesized that this SNP might have a functional effect on *FURIN* gene expression although the possibility that ≥1 of the SNPs in LD with rs17514846 might also be functional could not be precluded.

SNP rs17514846 Influences FURIN Expression

To investigate whether the CAD-associated variant at the 15q26.1 locus has an effect on the expression levels of *FURIN, FES*, *BLM*, *MAN2A2*, and *UNC45A*, we genotyped a group of individuals for SNP rs17514846 and performed quantitative

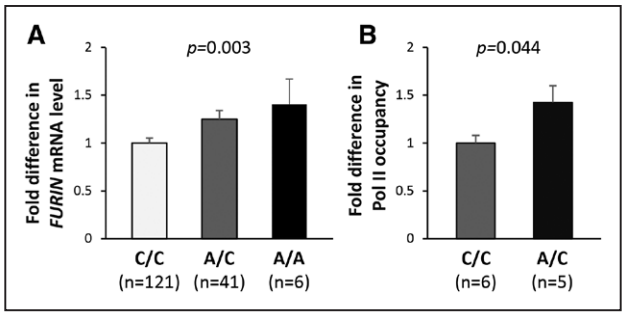

Figure 1. Influence of single nucleotide polymorphism rs17514846 genotype on FURIN expression. **A**, Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. Blood leukocytes from a group of individuals without abnormal coronary angiographic finding were genotyped for rs17514846 and subjected to quantitative RT-PCR analysis of FURIN. Shown in the graph is the fold difference (±SEM) between genotypes in FURIN mRNA level. **B**, Chromatin immunoprecipitation assay. Mononuclear cells from individuals of the C/C or A/C genotype for rs17514846 were subjected to chromatin immunoprecipitation using an antibody against RNA polymerase II (Pol II), followed by quantitative PCR analysis of the *FURIN* gene in the immunoprecipitated DNA. The column chart shows the fold difference between genotypes in the amount of Pol II–precipitated chromatin DNA at the FURIN locus. Columns and error bars in (**A**) and (**B**) represent mean and SEM values.

RT-PCR to determine the mRNA levels of *FURIN*, *FES*, *BLM*, *MAN2A2*, and *UNC45A* in blood leukocytes from these subjects. The assay showed that individuals of the A/A or A/C genotype had higher *FURIN* mRNA levels than those of the C/C genotype, and the differences remained significant after adjusting for age, sex, hypertension, and diabetes mellitus (Figure 1A), suggesting that either rs17514846 per se, or another SNP in LD with it, exerts an allele-specific effect on *FURIN* expression. There was also a trend toward lower *FES* mRNA levels but higher *BLM* and *UNC45A* levels in individuals of the A/A or A/C genotype; however, the differences were nonsignificant statistically (Figure V in the online-only Data Supplement).

To further characterize the effect of rs17514846 genotype, we examined whether there is a difference between the 2 alleles in RNA Pol II occupancy as mRNA synthesis during transcription of protein-coding genes in humans is performed by Pol II. To this end, blood mononuclear cells from CAD patients of the A/C or C/C genotype were subjected to chromatin immunoprecipitation using an anti-Pol II antibody, followed by quantification of the amounts of immunoprecipitated DNA. The experiments showed that there was higher Pol II occupancy in mononuclear cells from CAD patients of the A/C genotype than those from patients of the C/C genotype, indicating that either rs17514846 or another SNP in LD with it has an influence on transcriptional activity at this locus (Figure 1B).

To begin to investigate whether rs17514846 per se has an allele-specific influence on transcription, we performed a gene transcription reporter assay. Cultured monocytes/macrophages were transfected with plasmid constructs containing either the A or C allelic sequence upstream of a luciferase

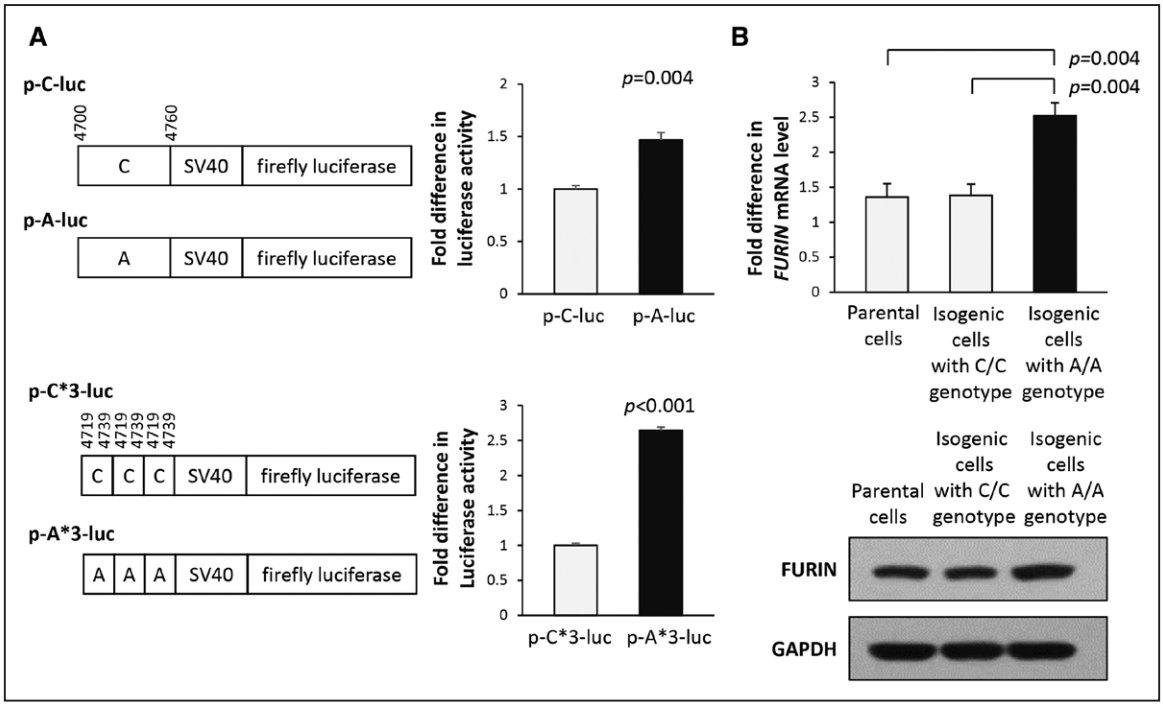

Figure 2. Allele-specific effect of single nucleotide polymorphism rs17514846 on FURIN expression. **A**, Luciferase reporter assay. Two sets of plasmids containing DNA sequences of the C or A allele of rs17514846 followed by a firefly luciferase reporter gene were generated, as shown on the left. Each of these plasmids, together with a plasmid containing a Renilla luciferase gene to serve as a transfection efficiency reference, was transfected into monocytes/macrophages, followed by measurement of activities of firefly luciferase and Renilla luciferase. The fold difference between the A allele construct and the C allele construct in firefly luciferase activity is shown in the column chart, with values standardized against Renilla luciferase activity. **B**, Analysis of isogenic cell lines generated by CRISPR (clustered regularly interspaced short palindromic repeats)-mediated genome editing. CRISPR-mediated genome editing on a monocyte cell line was performed to generate 2 isogenic cell lines with either the C/C or A/A genotype for rs17514846, followed by FURIN quantitative reverse transcription polymerase chain reaction (RT-PCR) and Western blot analyses. **Top**, results of quantitative RT-PCR analysis of FURIN in the isogenic cell lines; (**bottom**): images of Western blotting analysis of the isogenic cell lines, with the use of antibodies against FURIN and the housekeeping reference protein GAPDH, respectively. Columns and error bars in (**A**) and (**B**) represent mean and SEM values in 3 experiments.

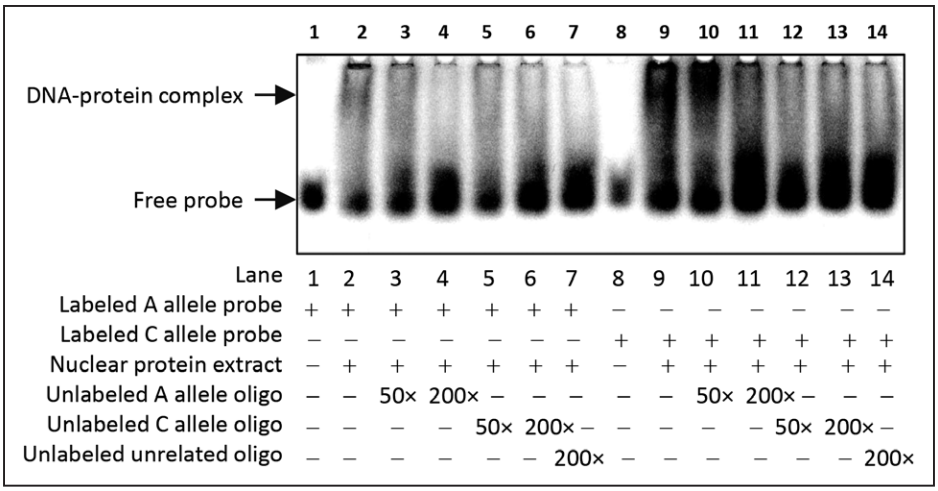

Figure 3. Allele-specific effect of single nucleotide polymorphism (SNP) rs17514846 on nuclear protein binding. Fluorescent FAM (fluorescein)-labeled double-stranded oligonucleotide probes corresponding to the sequence from nucleotide position –4717 to –4741 in the *FURIN* gene, with either A or C at the SNP rs17514846 site at position –4729, were individually incubated with or without monocyte/macrophage nuclear protein extracts, in the presence or absence of unlabeled A allele oligonucleotide, unlabeled C allele oligonucleotide, and an unrelated oligonucleotide, respectively, followed by nondenaturing polyacrylamide gel electrophoresis and fluorescence detection for the labeled probes. Lanes 1 and 8, without nuclear extracts; lanes 2 and 9, without unlabeled oligonucleotide; lanes 3 and 10, with unlabeled A allele oligonucleotide in 50-fold molar excess; lanes 4 and 11, with unlabeled A allele oligonucleotide in 200-fold molar excess; lanes 5 and 12, with unlabeled C allele oligonucleotide in 50-fold molar excess; lanes 6 and 13, with unlabeled C allele oligonucleotide in 200-fold molar excess; lanes 7 and 14, with unlabeled unrelated oligonucleotide in 200-fold more excess. Arrows indicate a DNA-protein complex and the free probe, respectively. Image shown is a representative from 3 experiments.

reporter gene, followed by luciferase assays. The experiment showed that the A allelic sequence had greater activity in driving the transcription of the reporter gene than the C allelic sequence (Figure 2A).

To substantiate that rs17514846 per se has an allele-specific effect on *FURIN* expression, we performed CRISPR-mediated genome editing on a monocytic cell line to generate 2 isogenic cell lines with either the C/C or A/A genotype for rs17514846. We then performed quantitative RT-PCR and Western blot analysis to determine and compare *FURIN* mRNA and protein levels in these isogenic cells. The experiment showed that isogenic cells with the A/A genotype had higher *FURIN* mRNA and protein levels than isogenic cells with the C/C genotype (Figure 2B), further supporting that

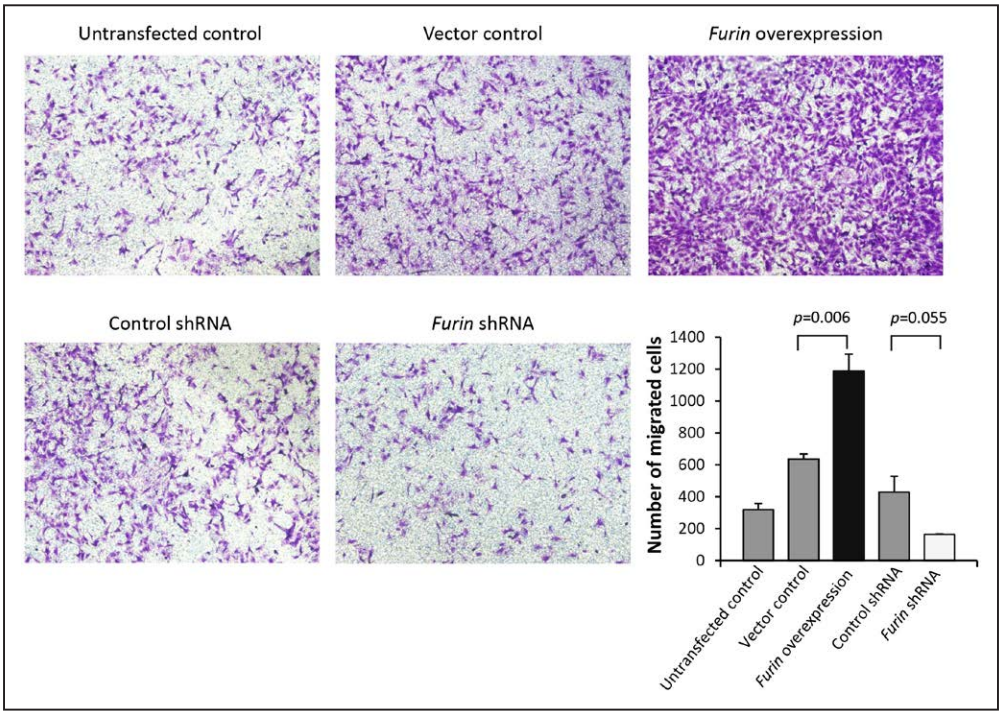

Figure 4. Effect of furin overexpression and knockdown on monocyte/macrophage migration. Monocytes/macrophages either without transfection or transfected with a control lentivirus vector or a lentivirus to overexpress furin or a control shRNA (short hairpin RNA) or a Furin shRNA were subjected to transwell migration assay. Cells that had migrated to the lower surface of the transwell were stained with crystal violet and counted under a microscope. Representative microscopic images are shown. The column chart shows mean numbers of migrated cells (and SEM as error bars) in 5 or 6 experiments.

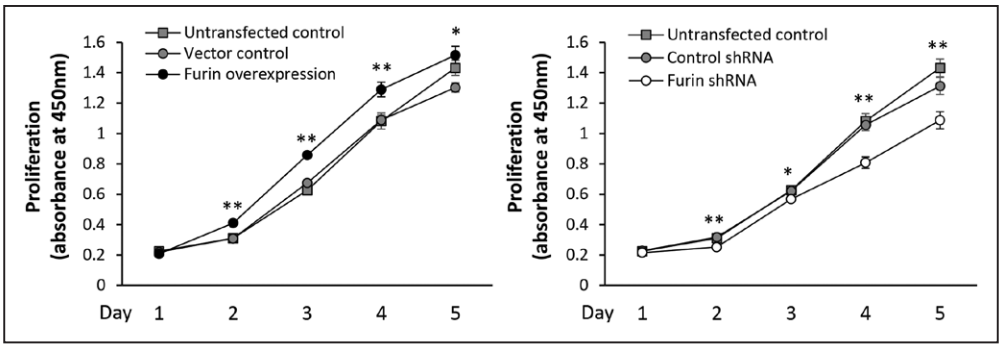

Figure 5. Effect of furin overexpression and knockdown on monocyte/macrophage proliferation. Monocytes/macrophages either without transfection or transfected with a control lentivirus vector or a lentivirus to overexpress furin or a control shRNA (short hairpin RNA) or a Furin shRNA were subjected to proliferation assay with the use of Cell Counting Kit-8. Proliferation rates were measured by the absorbance at 450 nm in colorimetric assays. Data shown are mean and SEM values in 6 experiments. **P*<0.05; ***P*<0.01.

rs17514846 itself has an allele-specific effect on *FURIN* expression.

Having found that rs17514846 has an allele-specific effect on gene transcription, we investigated whether this SNP is located at a nuclear protein binding site and has an allele-specific effect on binding. To this end, electrophoretic mobility shift assays were performed in which florescent FAM-labeled DNA probes corresponding to either the A or C allele were incubated with or without monocyte/macrophage nuclear protein extracts, in the presence or absence of unlabeled A allele oligonucleotide, unlabeled C allele oligonucleotide, and an unlabeled unrelated oligonucleotide, respectively, followed by nondenaturing polyacrylamide gel electrophoresis and florescence detection of the labeled DNA probes. In this experiment, a DNA-protein complex was detected with higher band intensity in the assays with the labeled C allele probe (lane 9, Figure 3) than in the assays with the labeled A allele probe (lane 2, Figure 3), indicating preferable binding of the nuclear protein to the C allele. This DNA-protein complex was barely detectable in the presence of the unlabeled C allele oligonucleotide as a competitor in 50× molar excess (lane 12, Figure 3) but was still detectable in the presence of the unlabeled A allele oligonucleotide in 50× molar

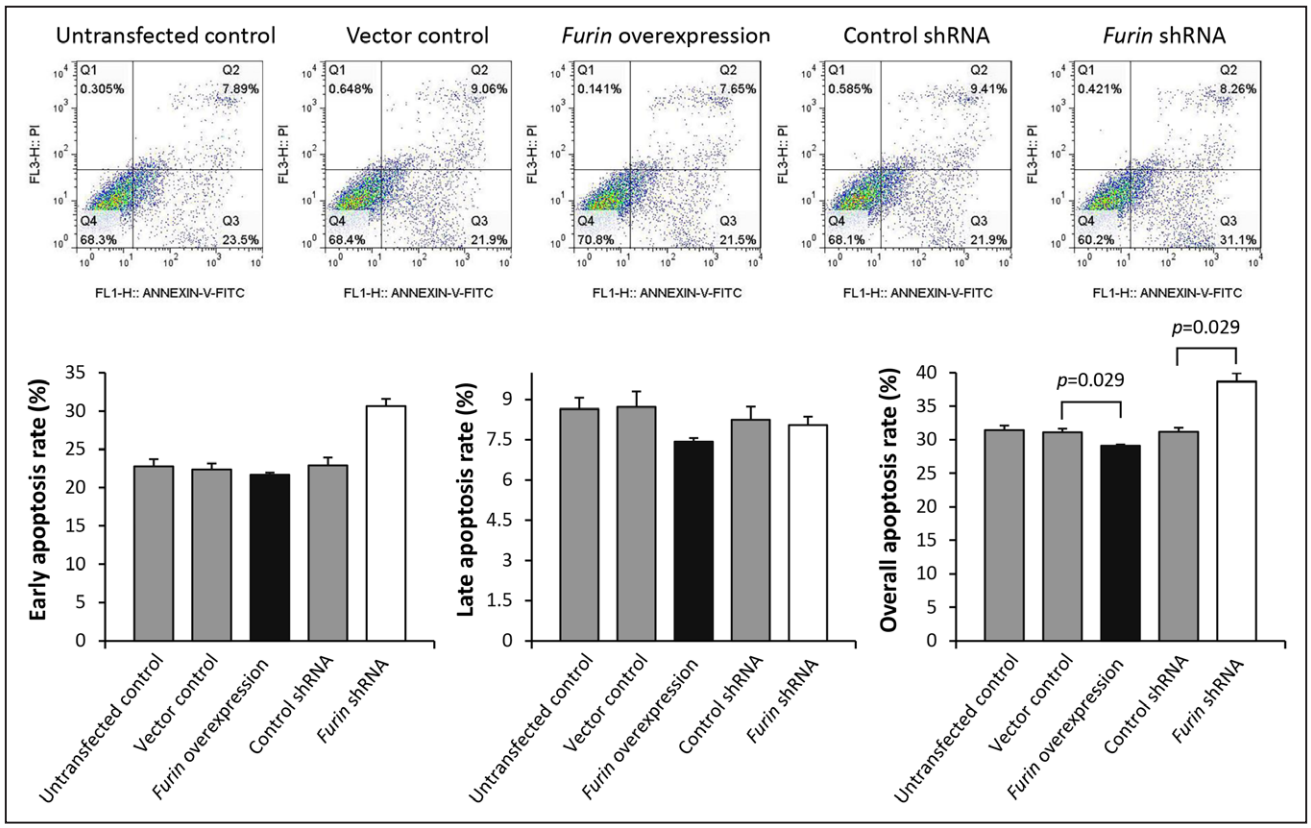

Figure 6. Effect of furin overexpression and knockdown on monocyte/macrophage apoptosis. Monocytes/macrophages either without transfection or transfected with a control lentivirus vector or a lentivirus to overexpress furin or a control shRNA (short hairpin RNA) or a Furin shRNA were subjected to apoptosis assay with the use of annexin V-fluorescein isothiocyanate/propidium iodide Apoptosis Detection kits and flow cytometry. The upper row shows representative flow cytometry images in which necrotic cells are in the Q1 area, late apoptotic cells in Q2, early apoptotic cells in Q3, and healthy cells in Q4. Data shown in the column charts in the lower row are mean and SEM values in 4 experiments.

excess (lane 10, Figure 3), further illustrating preferable binding of the nuclear protein to the C allele.

Furin Affects Monocyte/Macrophage Migration, Proliferation, and Apoptosis

To investigate possible effects of furin on monocyte/macrophage behavior, we tested whether overexpressing or knocking down furin in monocytes/macrophages would alter their migratory ability, proliferation rates, and apoptosis rates. To this end, we transfected cultured monocytes/macrophages with either a lentivirus to overexpress furin or a control lentivirus. In separate experiments, cultured monocytes/macrophages were transfected with either a furin shRNA or a control shRNA. Efficient furin overexpression or knockdown is shown in Figure II in the online-only Data Supplement. Transfected cells were subjected to migration assays by the transwell technique, proliferation assays using Cell Counting Kit-8, and apoptosis assays by flow cytometry with annexin V and propidium iodide staining, respectively.

The experiments showed that furin overexpression significantly increased the migratory ability of monocytes/macrophages whereas furin knockdown inhibited monocyte/macrophage migration (Figure 4). Furthermore, furin overexpression increased the proliferation rate of monocytes/macrophages while furin knockdown lowered their proliferation rate (Figure 5). Meanwhile, furin overexpression reduced while furin knockdown increased, monocyte/macrophage apoptosis (Figure 6).

Discussion

The association between the chromosome 15q26.1 locus and CAD has been replicated in several studies, with the minor allele (A) of SNP rs17514846 robustly associating with increased CAD risk.[1–4] However, the biological and pathological mechanism underlying this genetic association is hitherto unknown.

rs17514846 resides in intron 1 of the *FURIN* gene and is in LD (r^2>0.8) with several other SNPs nearby. A bioinformatics analysis with data from Encyclopedia of DNA Elements and Roadmap Epigenomics shows that rs17514846 resides in a transcriptionally active region (Figures III and IV in the online-only Data Supplement). One of the key findings of our present study is that rs17514846 has a direct, allele-specific effect on *FURIN* expression and that the CAD risk allele (A) has higher *FURIN* expression level than the nonrisk allele (C). However, it is possible that ≥1 of the SNPs in LD with rs17514846 might also have a functional effect.

The furin protein encoded by the *FURIN* gene is an important proprotein convertase. Newly synthesized furin is a proenzyme that undergoes autoactivation intracellularly.[19] The activated furin traffics to the trans-Golgi network/endosomal system and then localizes to the cell surface.[19] Active furin possesses proteolytic activity on many proteins, including several growth factors (TGFβ1 [transforming growth factor beta 1],[5] IGF1 [insulin-like growth factor 1],[6] and NGF [nerve growth factor][7]), metalloproteinases (MMP14 [matrix metalloproteinase],[8] ADAM10 [a disintegrin and metalloprotease],[9] ADAM17,[10] ADAMTS4,[11] and ADAMTS7[12]), and integrin-α(v).[20] By proteolytically processing these proteins, furin can activate these molecules, in turn leading to various biological consequences, such as changes in cell behavior.[21–23]

In this study, we found that the levels of furin in monocytes/macrophages have an effect on monocyte/macrophage behavior, such that an increased furin level results in increased monocyte/macrophage migration and proliferation and less apoptosis, whereas a reduced furin level has the opposite effect. This previously unreported function of furin is noteworthy because macrophages in atherosclerotic plaques express furin[13,14] and because monocytes/macrophages play important roles in inflammatory diseases, such as atherosclerosis.[15–17] Monocytes/macrophages are a key component of the atherosclerotic plaque. It is well established that the development and progression of the atherosclerotic plaque involve migration of monocytes from the bone marrow and spleen to the blood circulation and then into the arterial wall.[15–17] In addition, recent evidence suggests that macrophage proliferation is primarily responsible for macrophage accumulation in the atherosclerotic lesion.[17,24] Furthermore, studies have indicated that macrophage apoptosis suppresses early atherosclerotic lesion development and growth[25,26] but negatively affects advanced atherosclerotic plaque stability.[16,27]

A search in the GTEx Portal revealed that SNP rs17514846 is associated with *FURIN* expression in the aorta and the tibial artery. Thus, it is possible that it has an influence on *FURIN* gene expression not only in monocytes/macrophages but also in other cell types, such as vascular endothelial and smooth muscle cells. If this is the case, its association with CAD susceptibility may be a result of its effect on >1 cell type that plays a role in atherogenesis.

A previous study showed that rs17514846 is associated with the expression of the *FURIN*, as well as *FES* in monocytes.[18] In the present study, in addition to the finding that rs17514846 genotype has an influence on *FURIN* expression levels in blood leukocytes, we detected a tendency toward lower *FES* expression in blood leukocytes of individuals with the rs17514846 A/A or A/C genotype (Figure V in the online-only Data Supplement). Previous studies demonstrate that Fes regulates leukocyte recruitment during inflammation. Therefore, it is possible that the influence of genetic variation at this locus on CAD susceptibility is attributable to altered expression of >1 gene, including *FURIN*, *FES*, and possible also some other genes as such *BLM*, *MAN2A2*, and *UNC45A* which also located in this genomic region although no statistically significant difference between rs17514846 genotypes in the levels of *FES*, *BLM*, *MAN2A2*, and *UNC45A* was detected in this study.

In summary, our study shows that the CAD risk allele of SNP rs17514846 increases *FURIN* expression and that furin promotes monocyte/macrophage migration and proliferation while inhibiting apoptosis. Because monocyte/macrophage migration, proliferation, and apoptosis are key events in the pathogenesis of atherosclerosis, the pathology underlying CAD, the results of our study provide a plausible mechanistic explanation for the association between rs17514846 and CAD risk.

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Disclosures

None.

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Highlights

- The coronary artery disease–associated SNP rs17514846 has an allele-specific effect on *FURIN* expression.
- The coronary artery disease risk allele (A) of rs17514846 has higher *FURIN* expression level than the nonrisk allele (C).
- Increased furin expression promotes monocyte/macrophage migration.
- Increased furin expression induces monocyte/macrophage proliferation.
- Increased furin expression inhibits monocyte/macrophage apoptosis.