Coronary artery disease susceptibility gene ADTRP regulates cell cycle progression, proliferation and apoptosis by global gene expression regulation

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**ABSTRACT**

The *ADTRP* gene encodes the Androgen-Dependent TFPI-Regulating Protein and is a susceptibility gene for contrary artery disease (CAD). We performed global gene expression profiling for *ADTRP* knockdown using microarrays in human HepG2 cells. Follow-up real-time RT-PCR analysis demonstrated that *ADTRP* regulates a diverse set of genes, including up-regulation of 7 histone genes, down-regulation of multiple cell cycle genes (*CCND1, CDK4* and *CDKN1A*), and up-regulation of apoptosis genes (*CASP7* and *PDCD2*) in HepG2 cells and endothelial cells. Consistently, *ADTRP* increases the number of S phase cells during cell cycle, promotes cell proliferation and inhibits apoptosis. Our study provides novel insights into the function of *ADTRP* and biological pathways involving *ADTRP*, which may be involved in the pathogenesis of CAD.

**Keywords:** global gene expression microarray; coronary artery disease (CAD); *ADTRP*; histone; cyclin D1; CDK4; p21<sup>Clp</sup>; caspase 7
INTRODUCTION

Coronary artery disease (CAD) and its complication myocardial infarction (MI) are the leading cause of death worldwide (29-31). It is responsible for more than 400,000 sudden deaths in the USA and more than 700,000 deaths in China each year (16, 27). Our genome-wide association studies (GWAS) in the Chinese population identified a genomic variant, rs6903956, in intron 1 of the C6orf105 gene (later named as ADTRP) as a significant risk factor for CAD and MI (27). This finding was confirmed by multiple follow-up replication studies (11, 17, 19, 25). The ADTRP gene encodes a 230 amino acid protein referred to as androgen-dependent TFPI-regulating protein because it regulates expression of tissue factor pathway inhibitor (TFPI) in endothelial cells and its expression can be induced by androgen (22). ADTRP was found to be expressed in human macrophages and atherosclerotic lesions (9). Chinetti-Gbaguidi and co-authors further showed that activation of PPARγ (Peroxisome Proliferator-Activated Receptor) up-regulated macrophage expression of ADTRP in vitro and in vivo in human atherosclerotic lesions (9).

Based on the UniProtKB database (entryQ96IZ2), the ADTRP protein is a cell membrane protein with 6 transmembrane domains (amino acid residues 4-27, 47-67, 86-106, 120-140, 155-175, and 190-210). It co-localizes with TFPI and CAV1 in lipid rafts (22). Based on its cell membrane localization and its function on regulation of TFPI, we hypothesize that ADTRP acts as a cell signaling molecule that affects function and expression of many downstream genes/proteins. To identify other downstream targets of ADTRP, we performed global gene expression profiling in cells with knockdown of ADTRP expression. Because ADTRP downstream genes include those involved in cell cycle regulation and apoptosis as
well as multiple histone genes, we carried out cellular studies on cell cycle, cell proliferation
and apoptosis to further characterize the function of \textit{ADTRP}.

\textbf{MATERIALS AND METHODS}

\textit{Plasmids and siRNAs.} A plasmid for \textit{ADTRP} (UniProtKB - Q96IZ2-3, alternatively
spliced isoform 3), PUC57-ADTRPiso3, was purchased from GenScript. The isoform 3
transcript was the longest \textit{ADTRP} transcript in the GeneBank database and encodes an
\textit{ADTRP} protein with 255 amino acid residues. The full length cDNA for \textit{ADTRP} isoform 3
was amplified by PCR analysis using PUC57-ADTRP as a template and primers
ADTRP(255aa)768bp F-XbaI: GCTCTAGAatgacgaagacttctacatgcatatacc and
ADTRP(255aa)768bp R-EcoRI: CCGGAATTCtcacctgatattcttgaccagttgg. The PCR product
was digested with \textit{XbaI} and \textit{EcoRI} (TAKARA) and sub-cloned into the multiple cloning site
of eukaryotic expression vector pCDNA3.1(-), resulting in an expression plasmid for \textit{ADTRP},
pCDNA3.1(-)-ADTRPiso3.

Recently, a short isoform of \textit{ADTRP} mRNA was denoted as the 'canonical' sequence
(referred to as isoform 1, Q96IZ2-1) and encodes an \textit{ADTRP} protein with 230 amino acid
residues. Thus, we also created a mammalian expression plasmid for the canonical isoform
of \textit{ADTRP}, pCDNA3.1(-)-ADTRPiso1. The isoform 1 \textit{ADTRP} transcript was directly
synthesized and cloned into PUC57, resulting in PUC57-ADTRPiso1. The isoform 1
\textit{ADTRP} transcript was then amplified by PCR using PUC57-ADTRPiso1 as the template and
primers ADTRP(230aa)693bpF-Xbal: GCTCTAGAatgacgaagacttctactcatgacttttaccac and
ADTRP(230aa)693bp R-EcoRI: CCGGAATTCTactctacactt tacatgttgccggtgtg. The PCR product
was digested with *Xba I* and *EcoR I* (TAKARA) and sub-cloned into the multiple cloning site of eukaryotic expression vector pCDNA3.1(-), resulting in an expression plasmid for *ADTRP*, pCDNA3.1(-)-ADTRPiso1. Identical results were obtained for pCDNA3.1(-)-ADTRPiso3 and pCDNA3.1(-)-ADTRPiso1, thus we later referred pCDNA3.1(-)-ADTRP as either pCDNA3.1(-)-ADTRPiso3 or pCDNA3.1(-)-ADTRPiso1 in the text.

The PCR products were also digested with *EcoR I* and *Xho I* (TAKARA) and sub-cloned into the multiple cloning site of eukaryotic tag expression vector pCMV-Myc, which generated eukaryotic expression plasmids pCMV-Myc-ADTRPiso3 and pCMV-Myc-ADTRPiso1.

Small interfering RNA (siRNA) specific to human *ADTRP* (*ADTRP* siRNA) and negative control siRNA (NC siRNA) were purchased from Genepharma. The sequences of *ADTRP* siRNA were (5'-GGAUCCUCUUUCUCUACAATT-3' (sense) and (5'-UUGUAGAGAAAGAGGAUCCTT-3' (antisense). The sequences of NC siRNA were (5'- UUCUCCGAACGUGACACGUUATT-3' (sense) and (5'-ACGUGACACGUUCCGAAGAATT-3' (antisense).

**Cell culture and transfection.** A HepG2 cell line was purchased from ATCC (American Type Culture Collection) and maintained in the Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Giboc, Thermo Fisher Scientific). Human umbilical vein endothelial cells (HUVECs) were purchased from Pricells and maintained in the human endothelial basal growth medium supplemented with 10% FBS. EAhy926 endothelial cells were purchased from Shanghai Institute of Biochemistry and Cell Biology of SIBCB and maintained in the human endothelial basal growth medium.
supplemented with 10% FBS. All cells were cultured at 37°C in a humidified incubator with 5% CO₂.

Transfection of plasmid DNA (1 µg) was carried out using Lipofectamine®2000 (2 µl) according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific).

Transfection of siRNA (80 nM) was performed using Lipofectamine® RNAi MAX according to the manufacturer’s protocol (Invitrogen, Thermo Fisher Scientific). For endothelial cell studies, we used HUVEC for siRNA analysis, but used EAhy926 endothelial cells when transfection is needed for plasmid DNA because the transfection efficiency for HUVEC was too low to perform a study.

*The GeneChip® PrimeView™ human gene expression array analysis.* Microarray analysis was carried out as described by us previously (1, 2, 4). HepG2 cells were transfected with ADTRP siRNA or NC siRNA (80 nM) using Lipofectamine® RNAi MAX and incubated for 48 hours. Total RNA samples were isolated using the Trizol reagent according to the manufacturer’s instruction (Takara Bio Inc.), and purified by using RNeasy Mini Kit (Qiagen). All purified RNA samples passed initial quality control. RNA integrity number (RIN) ranged from 9.1 to 9.8 and the ratio of 28s/18s was between 1.7 and 2.1.

Each RNA sample (25 µg) was then used to generate biotinylated cRNA targets for the Gene Chip® Prime View™ Human Gene Expression Array, which contains more than 49,000 expression probes, providing comprehensive coverage of all well-annotated genes. The biotinylated cRNA targets were hybridized with microarrays. After hybridization, arrays were stained in the Fluidics Station 450 and scanned on the Affymetrix Scanner3000. The microarray experiments and genome-wide expression quantification were performed by
following the protocol of Affymetrix Inc at Shanghai Biotechnology Corporation. Raw probe intensities were further normalized by Robust Multi-array Average (RMA) algorithm, which was implemented by Gene Spring Software 11.0 (Agilent Technologies). A total of 49,293 expression probes with intensities distinguishable from background noise (detection $P$ value $<0.05$) in all RNA samples were filtered out for downstream differential analysis. Compared with NC siRNA group, probes or genes showing differential expression levels in the $ADTRP$ siRNA group were identified by Student’s $t$ tests. Top genes with $P<0.01$, fold change $>2$, and average expression levels of greater than 7 (usually ranging from 2 to 14) were selected for validation analysis. The selection resulted in a total of 120 probes/genes for validation analysis. To adjust for multiple testing, we used the false discovery rate (FDR) method in the R package (http://github.com/jdstorey/qvalue), and computed $Q$ values for the 120 probes, i.e. adjusted $P$ values after controlling the FDR at 0.05.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (5, 12) and are accessible through GEO Series accession number GSE80469 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE80469).

*Gene set enrichment tests.* The Heatmap plot was generated to show normalized expression levels of top 120 $ADTRP$ siRNA targeted probes or genes among 6 RNA samples. Hierarchical clustering analysis built-in R package, “gplots”, was applied to show dissimilarities between top 120 genes (left-side dendrogram) across 6 RNA samples (top dendrogram). To explore potential functional implications of top differentially expressed genes, top 120 expression probes were mapped to 94 unique genes based on the Affymetrix annotation database. A set of 94 unique genes were then uploaded to program “ConceptGen”
(http://conceptgen.ncibi.org/core/conceptGen/index.jsp), which is an online gene set enrichment analysis tool to identify significant overlap among massive sets of genes (referred to as “concept” in ConceptGen) and to visualize their interconnections. ConceptGen collects gene set data retrieved from diverse areas such as Gene Ontology (GO) Biological process and Cellular Functions (3), Protein Domains (Pfam) (14), Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, literature derived Medical Subject Headings (MeSH), and protein-interaction databases (http://mimi.ncibi.org/MimiWeb/main-page.jsp). A significant gene set or concept was defined as one with an observed \( P \) value of < 0.01 as calculated by modified Fisher’s exact test (18) and a \( Q \) value of < 0.05 as calculated by the FDR method (6).

Quantitative reverse-transcription PCR (qRT-PCR) analysis. The qRT-PCR analysis with independent sets of RNA samples was used for validation of the microarray data. Cells were transfected with \( ADTRP \) siRNA vs. NC siRNA or pcDNA3.1-ADTRP vs. control pcDNA3.1 vector as described above and incubated at 37°C for 48 hours. Cells were then collected for preparation of total RNA samples using the Trizol reagent according to the manufacturer's instruction (Takara Bio Inc.). Total RNA samples were treated with DNase I to remove contaminating genomic DNA and then converted into cDNA by reverse transcription with M-MLV reverse transcriptase using random primers (Promega). The cDNA was then analyzed by real-time quantitative PCR (qRT-PCR) with Fast Start SYBR Green Master (Roche Applied Science).

For all 94 unique, downstream target genes of \( ADTRP \) identified by microarray analysis, we designed primers for qRT-PCR analysis. The primers were successful for qRT-PCR
analysis of 71 genes, 38 down-regulated genes and 33 up-regulated genes with ADTRP knockdown. These 71 genes were then selected for follow-up validation. The sequences of qRT-PCR primers for the 71 genes are shown in Supplemental Table S1.

Real-time PCR was performed in a 10 μl reaction volume containing 5 μl of SYBR green I mix, 200 mM of forward and reverse primers and 1 μl cDNA template on an ABI 7900 Genome Analyzer System. The PCR profile was 95°C for 5 min, and 45 cycles of 95°C for 15 s and 60°C for 20 s. The PCR products were verified by melting curve analysis as well as by 2.0% agarose gel electrophoresis. GAPDH was used as an internal control. The data were analyzed using the method of $2^{-\Delta\Delta Ct}$ relative expression quantity as previously described (7, 8, 21, 33, 34). Fold changes in expression were calculated. All the qRT-PCR reactions were run in triplicate and each experiment was repeated at least three times.

**Cell cycle assays.** For the cell cycle analysis, cells were transfected as described above, and transfected cells were harvested by trypsinization, washed twice using cold PBS and fixed in 70% ethanol over night at -20°C. Cells were then treated with 3.4 mM Tris-Cl (pH7.4) and 0.1% tritonX-100 for 20 min, and 100 mg/ml RNaseA for 30 min, followed by treatment with propodium iodide. Cell cycle analysis was performed with a Beckman Coulter Cytomics FC500 flow cytometry and CXP software (Beckman Coulter). Each experiment was repeated at least three times.

**Cell proliferation assays.** Cells were transfected as described above. Transfected cells (5×10^3 per well) were plated in 96-well plates and co-transfected on the following day with 80 nM ADTRP siRNA (or 80 nM NC siRNA) and 125 ng of plasmid PCDNA3.1(-)ADTRP (or 125 ng of empty vector PCDNA3.1(-)) using Lipofectamine®2000 (Invitrogen, Thermo
Fisher Scientific). Cell proliferation was determined at 24 hr, 48 hr, and 72 hr using Cell Counting Kit-8 (CCK8) according to the manufacturer's instruction (Dojindo). The absorbance representing the number of living cells was measured at 450 nM using a VersaMax Absorbance Microplate Reader (Molecular Devices). The assays were performed in triplicate and repeated at least three times.

Apoptosis assays. Cells were transfected as described above. Transfected cells were harvested and used for apoptosis assays using a Beckman Coulter Cytomics FC500 flow cytometry and CXP software (Beckman Coulter). Briefly, cells were treated with annexinV-FITC and propidium iodide (PI) from an Apoptosis Detection Kit (BD). AnnexinV-FITC-positive cells were counted as early apoptotic cells. Cells that were positive for both annexinV-FITC and PI were counted as late apoptotic cells. Viable cells were negative for both annexinV-FITC and PI. Each experiment was repeated at least three times.

Western blot analysis. HepG2 and EAhy926 cells were transfected as described above, harvested after 48h, and lysed in RAPI lysis buffer (50 mM Tris, pH7.4, 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 0.1% SDS) (Beyotime) with the protease inhibitor cocktail (Roche). The cell extracts were used for Western blot analysis with a rabbit polyclonal antibody against p21\textsuperscript{CIP} (1:1000 dilution, Epitomics) or a control mouse monoclonal antibody against \(\alpha\)-tubulin (1:5000 dilution, Millipore) as described (13, 32). Each experiment was repeated at least three times.

Statistical analysis. All data were presented as means ± standard error of means (SEM). Each experiment was repeated at least three times. Statistical analysis was performed for comparison of mean values between two groups using a Student’s t-test with SPSS version
16.0 software (SPSS). A $P$ value of <0.05 or less was considered to be statistically significant.

RESULTS

Microarray analysis to identify downstream target genes regulated by ADTRP. Because of high efficiency of transfection, HepG2 cells were studied in all initial experiments including global gene expression microarray analysis. Then, major findings from HepG2 cells were replicated in HUVEC and EAhy926 endothelial cells because $ADTRP$ was highly expressed in endothelial cells (22) and dysfunction of endothelial cell function plays an important role in the development of CAD and MI and can predict cardiovascular outcomes independent of conventional risk factors (2, 28-30). Compared with 3 NC siRNA samples, the expression levels of 120 expression probes were identified to be significantly altered in 3 $ADTRP$ siRNA samples with a $P$ value of less than 0.01, more than 2 fold changes and average expression levels of greater than 7.0. The normalized expression levels of the identified 120 probes were shown in Fig. 1A. Hierarchical clustering analysis revealed that top 120 probes formed 6 types of expression patterns, including 3 up-regulated patterns and 3 down-regulated patterns. The 6 samples were clustered into 2 distinct subgroups that perfectly matched their treatment status ($ADTRP$ siRNA vs. NC siRNA). Among the 120 probes, 106 probes were mapped to one unique gene while 14 probes were mapped to more than one gene, leveraging 94 unique genes for gene set enrichment analysis. Program ConceptGen discovered about 12 gene sets (so called “concepts”) that were significantly over-represented by $ADTRP$ siRNA top targets (Table 1). The highly enriched gene sets
included histones (8 overlapping genes, $P = 6.5 \times 10^{-7}$, $Q = 1.1 \times 10^{-3}$) and nucleosome assembly (6 overlapping genes, $P = 1.5 \times 10^{-4}$, $Q = 7.9 \times 10^{-4}$) (Table 1). Of interest, the identified gene sets were connected with each other (Fig. 1B), indicating that they share a common set of genes. For example, histone genes can execute biological functions related to nucleosome, histone acetylation, chromatin assembly and chromosomes (Fig. 1C, Table 1). ConceptGen also identified other gene sets such as mesenchymal stem cells, protein-serine-threonine kinases, glycogen synthase kinase 3, E2F1 interactions, hsa-miR-373*, AKT2 interactions, and others, but they did not survive FDR correction ($P < 0.01$, $Q > 0.05$).

We applied principle component analysis to visualize sample-wise distance using global expression data, and found that 6 microarray samples belonged to 2 distinct experimental groups as expected (Fig. 2A). Because low abundance genes are difficult to be detected, we examined dispersion (represented by coefficient of variance) of all 49,293 expression probes or genes. Compared with moderate or high abundance genes (i.e., expression level > 7), larger variance was observed on low abundance genes (Fig. 2B). Thus, we chose an expression level of 7 as the cutoff threshold for microarray analysis. In order to reduce false positive signals, besides the simple $P$ value rule, we incorporated 2 more parameters, 2-fold changes and average expression levels of 7, to prioritize selection of top genes. We also adjusted $P$ values for multiple testing using FDR correction ($Q$ values) (Supplemental Table S2-5). Among the top 120 probes, 118 probes survived FDR correction ($Q \leq 0.05$) and two probes yielded marginal $Q$ values of 0.06, including the probe for STAM2 (Supplemental Table S2) and one of the two probes for IF144L (data not shown).
The majority of the *ADTRP* downstream genes identified by microarray analysis were independently confirmed to be regulated by *ADTRP* by real-time RT-PCR analysis (Supplemental Tables S2-5). It is interesting to note that the data from microarray analysis fully matched the results from real-time RT-PCR analysis with regard to the directionality of changes, but the fold change of expression differed from a gene to another. Every gene selected for validation was confirmed. Sixty-three probes were found to be down-regulated by *ADTRP* knockdown, whereas 57 probes showed up-regulation after *ADTRP* knockdown (Fig. 1A). We selected 38 down-regulated genes and 33 up-regulated genes for validation using real-time RT-PCR analysis (Supplemental Tables S2-5). All 38 down-regulated genes were confirmed to be down-regulated by *ADTRP* knockdown in HepG2 and HUVEC cells (Supplemental Table S2). Consistently, expression of these 38 genes was up-regulated with *ADTRP* overexpression in HepG2 and EAhy926 endothelial cells (Supplemental Table S3). Thirty-three genes were confirmed to be up-regulated by *ADTRP* knockdown by real-time RT-PCR analysis HepG2 and HUVEC cells (Supplemental Table S4). Consistently, expression of these 33 genes was down-regulated with *ADTRP* overexpression in HepG2 and EAhy926 endothelial cells (Supplemental Table S5).

*ADTRP* regulates expression of histone genes. Our microarray analysis revealed that *ADTRP* regulated expression of 7 histone genes. We validated the microarray data using real-time RT-PCR analysis. Compared with NC siRNA, *ADTRP* siRNA significantly increased expression of 7 histone genes by >2 fold (Fig. 3A). In particular, the expression of *HIST1H2BJ* was increased by 4.78 fold by *ADTRP* siRNA (Fig. 3A). Compared with cells transfected with pcDNA3.1(-), cells with overexpression of *ADTRP* (the
pcDNA3.1(-)-ADTRP group) significantly reduced expression of the 7 histone genes (Fig. 3B).

Similar results were obtained in endothelial cells. HUVEC transfected with ADTRP siRNA showed a significantly higher expression of histone genes than cells with NC siRNA (Fig. 3C). EAhy926 cells transfected with pcDNA3.1(-)-ADTRP showed a significantly lower expression of histone genes than cells with pcDNA3.1(-) (Fig. 3D).

**ADTRP regulates cell cycle progression.** Cell cycle progression is a vital process by which the cell monitors its growth and differentiation. Gene expression microarray analysis revealed that ADTRP regulates expression of several key cell cycle genes, including CCND1 encoding cycling D1 and CDK4 encoding cyclin-dependent kinases 4 (CDK4) as well as CDKN1A encoding p21CIP (Supplemental Table S2). Follow-up qRT-PCR analysis showed that knockdown of ADTRP expression by siRNA significantly decreased expression of CCND1, CDK4 and CDKN1A in HepG2 cells (Fig. 4A) and HUVEC (Fig. 4C), whereas the expression of these three genes was significantly increased by overexpression of ADTRP in HepG2 cells (Fig. 4B) and EAhy926 cells (Fig. 4D). Western blot analysis further confirmed the qRT-PCR results on CDKN1A/ p21CIP (Fig. 5). Other ADTRP downstream genes related to cell cycle regulation are listed in Table 2.

Cyclin D1 and CDK4 form the active cyclin D1-CDK4 complex, which determines a cell's progress through the cell cycle (10, 15). Therefore, we hypothesize that ADTRP regulates cell cycle progression. To test the hypothesis, we determined the effect of ADTRP knockdown or overexpression on the change of the number of cells at the S phase during cell division. When ADTRP expression was knocked down in HepG2 cells using an
ADTRP-specific siRNA, the number of cells at the S phase significantly decreased compared with control NC siRNA (29.8% vs. 18.7%, \(P<0.001\), Fig. 6A and 6C). We also over-expressed ADTRP in HepG2 cells by transfection with pcDNA3.1(-)-ADTRP and found that cells with overexpression of ADTRP had a significantly increased number of cells at the S phase compared with cells transfected with negative control pcDNA3.1(-) (32.9% vs. 27.1%, \(P<0.001\), Fig. 6B and 6C). These data suggest that ADTRP regulates cell cycle progression. The changes of cell numbers at other phases of cell cycle are shown in Fig. 6A.

Similar results were obtained in EAhy926 endothelial cells. Knockdown of ADTRP expression decreased the number of cells at the S phase (10.4% +/- 0.43% for ADTRP siRNA vs. 29.3% +/- 1.17% for NC siRNA, \(P<0.001\), Fig. 6D and 6F). Overexpression of ADTRP increased the number of cells at the S phase (31% +/- 0.36% for pcDNA3.1(-)-ADTRP vs. 26% +/- 0.41% for pcDNA3.1(-), \(P<0.001\), Fig. 6E and 6F).

ADTRP regulates cell proliferation. Because ADTRP regulates cell cycle progression as demonstrated above, it is predicted to affect cell proliferation. Cell proliferation was significantly inhibited by ADTRP siRNA compared with NC siRNA (Fig. 7A). As shown in Fig. 7B, overexpression of ADTRP significantly increased cell proliferation at all three time points.

Similar results were obtained in EAhy926 endothelial cells. Overexpression of ADTRP significantly increased cell proliferation (Fig. 7C). Knockdown of ADTRP expression significantly decreased cell proliferation (Fig. 7D).
ADTRP regulates apoptosis. Gene expression microarray analysis revealed that ADTRP regulates expression of several genes involved in apoptosis, including CASP7 encoding caspase 7 and PDCD2 encoding programmed cell death protein 2 (Supplemental Table S4). Follow-up real-time RT-PCR analysis showed that knockdown of ADTRP expression by siRNA significantly increased expression of CASP7 and PDCD2 in HepG2 cells (Fig. 8A) and HUVEC (Fig. 8C), whereas the expression of these two genes was significantly decreased by overexpression of ADTRP in HepG2 cells (Fig. 8B) and EAhy926 cells (Fig. 8D). Therefore, we hypothesize that ADTRP regulates apoptosis. To test the hypothesis, we determined the effect of ADTRP knockdown or overexpression on apoptosis. ADTRP knockdown with ADTRP-specific siRNA significantly increased the rate of apoptosis compared with NC siRNA in HepG2 cells (Fig. 9A and 9C) and EAhy926 endothelial cells (Fig. 9D and 9F). In contrast, overexpression of ADTRP significantly decreased apoptosis in HepG2 cells (Fig. 9B and 9C) and EAhy926 cells (Fig. 9E and 9F).

DISCUSSION

In this study, using global gene expression microarray analysis we found that ADTRP, a susceptibility gene for CAD, regulates expression of a diverse set of downstream genes. Our follow-up analysis confirmed that ADTRP up-regulates 38 genes and down-regulates 33 genes. However, the underlying molecular mechanisms remain to be defined in future studies. As ADTRP is a plasma membrane with 6 transmembrane domains, it may act as a signaling protein that transmits the cell surface signal to the nucleus where transcription occurs. Interestingly, ADTRP down-regulates the expression of 7 histones genes (Fig. 3).
Histone proteins are required for the packaging of DNA into chromatin (26). An approximately 150 bp DNA is wrapped around a protein complex with two molecules each of the H2A, H2B, H3 and H4 core histone proteins, forming nucleosomes, the basic subunit of eukaryotic chromatin (26). Histone H1 links nucleosomes to form higher order structures, and further condensing generates chromosomes (26). Histones undergo extensive posttranslational modifications, including methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citrullination, and ADP-ribosylation (26). Regulation and modifications of histones play fundamental roles in many biological processes, including transcription, DNA damage repair, DNA replication, and recombination (26). Histone modifications have been studied extensively. Studies of regulation of histone expression were mostly focused on post-transcriptional control (20, 23, 24). Transcriptional control of histone genes is less studied, although three proteins, NPAT, HIRA and YY1 were shown to be involved in transcription of histone genes (20, 23, 24). In this study, we have demonstrated that \textit{ADTRP} regulates expression of 7 histone genes/proteins which belong to the categories of H2A, H2B, H4 and H1. Interestingly, \textit{ADTRP} acts as a suppressor of histone expression. Knockdown of \textit{ADTRP} expression increased expression of histone genes, whereas overexpression of \textit{ADTRP} reduced their expression (Fig. 3). The molecular mechanism by which \textit{ADTRP} regulates histone expression, however, remains to be identified. Nevertheless, identification of a cell surface protein, ADTRP, that regulates histone gene expression may aid in elucidation of regulation of histone gene expression during cellular signaling.
We found that *ADTRP* is pro-survival of HepG2 and endothelial cells. Knockdown of
*ADTRP* expression increased apoptosis, whereas overexpression of *ADTRP* reduced
apoptosis (Fig. 9). *ADTRP* may inhibit apoptosis by decreasing expression of *CASP7* and
*PDCD2* genes involved in apoptosis (Fig. 8).

We found that *ADTRP* promotes proliferation of HepG2 and endothelial cells.
Knockdown of *ADTRP* expression decreased cell proliferation, whereas overexpression of
*ADTRP* increased cell proliferation (Fig. 7). The molecular mechanism by which *ADTRP*
promotes cell proliferation is probably by increasing expression of cyclin D1 and CDK4 (Fig.
4). We also found that *ADTRP* increases expression of p21<sup>CIP</sup> (Fig. 4-5). Increased p21<sup>CIP</sup>
expression should inhibit the activity of cyclin-CDK complexes, resulting in inhibition of cell
proliferation (15). However, because cyclin D1 and CDK4 are downstream of p21<sup>CIP</sup> (10)
and *ADTRP* increases expression of cyclin D1 and CDK4, *ADTRP* enhances cell proliferation
even though p21<sup>CIP</sup> expression is also increased.

There are several limitations with the present study. First, our microarray analysis was
performed with a small sample size of three per group, which may lead to false negatives and
false positives. The false positives can be eliminated by follow-up validation studies;
however, the issue of false negatives can be resolved only by future studies with large sample
sizes. Second, in addition to endothelial cells, *ADTRP* was recently found to be expressed
in macrophages, which are also relevant to the pathogenesis of CAD (9). Future studies will
examine the effect of *ADTRP* overexpression or knockdown on the functions of macrophages.
Considering our findings that *ADTRP* had similar functions in HepG2 and endothelial cells, it
is likely that similar observations on cellular functions will be made in macrophages.

In summary, we have demonstrated that ADTRP is a pro-survival factor that promotes cell proliferation, which correlates with increased expression of cyclin D1 and CDK4, and inhibits apoptosis, which correlates with decreased expression of caspase 7 and PDCD2 in two cell lines examined. As knockdown of ADTRP expression increases apoptosis and decreases cell proliferation of endothelial cells, it will result in endothelial dysfunction, which serves as a substrate for the development of CAD and MI.

GRANTS

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DISCLOSURES

A related research project in Wang Laboratory received funding from Bayer Health.
APPENDIX A. SUPPLEMENTARY DATA

Supplementary data (Supplemental Tables S1-5) associated with this article can be found at the online version of the article.

AUTHOR CONTRIBUTIONS

Q.K.W., C.L., Q.C., conception and design of research; C.L., F.W., and S.Q. performed experiments; C.L., F.W., Q.C., Q.K.W. analyzed data and interpreted results of experiments; Q.K.W. and C.L. drafted manuscript; Q.K.W., C.L., Q.C. and F.W. edited and revised manuscript; Q.K.W. supervised the entire project.

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Figure legends:

Fig. 1. Identification of downstream genes regulated by ADTRP by microarray analysis. (A) Heatmap plot of 3 ADTRP siRNA treated samples and 3 NC siRNA treated samples using relative expression levels of top 120 probes. Each row represents one targeted probe or gene, which was defined with a cutoff \( P \) value of <0.01, >2-fold changes compared with NC and average expression levels of >7.0. The 6 samples (shown as columns) were clustered into 2 distinct subgroups that matched their treatment status. (B) A network diagram demonstrating top highly enriched gene sets. A set of 94 unique genes mapped by top 120 probes were uploaded to the online program “ConceptGen”, an enrichment test built-in “ConceptGen”, which revealed 12 significant gene sets with both a \( P \) value of <0.01 and a \( Q \) value of <0.05. Colors of circles indicated that 34 gene sets were annotated by several major annotation resources such as GO biological process, GO cellular components, protein interactions, gene expression databases, and pfam. Edge thickness indicated the number of overlapping genes. (C) Heatmap of overlapping genes versus selected gene sets. The left panel displayed all identified genes/gene sets. The right panel showed zoomed view for key genes/gene sets highlighted in the boxed area (top, genes; right, gene sets).

Fig. 2. Genome-wide expression data visualization. (A) Pair-wise distance between 3 ADTRP siRNA samples and 3 negative controls inferred by genome-wide expression data. X-axis and Y-axis represent the first two principle components calculated by principle component analysis. (B) Dispersion pattern of all 49,293 expression probes or genes. As shown in
Y-axis, the dispersion was demonstrated by coefficient of variation (CV), which was the ratio of standard deviation over average expression levels (X-axis).

Fig. 3. Effect of knockdown or over-expression of ADTRP on regulation of histone genes by quantitative real time RT–PCR analysis. The mRNA samples were prepared from transfected HepG2 or EAhy926 cells and used for measuring mRNA expression levels of 7 histone genes. GAPDH was used as a control for normalization. (A) Seven histone genes were significantly up-regulated by ADTRP knockdown in HepG2 cells. (B) Seven histone genes were significantly down-regulated by ADTRP overexpression in HepG2 cells. (C) Seven histone genes were significantly up-regulated by ADTRP knockdown in EAhy926 cells. (D) Seven histone genes were significantly down-regulated by ADTRP overexpression in EAhy926 cells. ***: $P<0.001$; **: $P<0.01$; *: $P<0.05$.

Fig. 4. Effect of knockdown or over-expression of ADTRP on regulation of cell cycle regulatory genes CDKN1A (p21CIP1), CCND1 (cyclin D1), and CDK4 (CDK4) by real time RT–PCR analysis. The mRNA samples were prepared from transfected HepG2 or EAhy926 cells and used for measuring mRNA expression levels of 3 genes involved in cell cycle progression and cell proliferation. GAPDH was used as a control for normalization. (A) Expression of CDKN1A, CCND1, and CDK4 was significantly down-regulated by ADTRP knockdown in HepG2 cells. (B) Expression of CDKN1A, CCND1, and CDK4 was significantly up-regulated by ADTRP overexpression in HepG2 cells. (C) Expression of CDKN1A, CCND1, and CDK4 was significantly down-regulated by ADTRP knockdown in EAhy926 cells. (D) Expression of CDKN1A, CCND1, and CDK4 was significantly up-regulated by ADTRP overexpression in EAhy926 cells. ***: $P<0.001$; **: $P<0.01$; *: $P<0.05$. 
Fig. 5. Western blot analysis showing regulation of p21CIP expression by ADTRP knockdown (A, D, G, J) or overexpression (B, E, H, K) in HepG2 cells (A, B, D, E) or EAhy926 endothelial cells (G, H, J, K). ADTRP siRNA was shown to be successfully knock the expression of ADTRP down in HepG2 cells (C, F) and EAhy926 endothelial cells (I, L) compared to negative control (NC) siRNA. Transfection of an expression plasmid pcDNA3.1(-)-ADTRP (ADTRP) led to successful overexpression of ADTRP in HepG2 cells (C, F) and EAhy926 endothelial cells (I, L) compared to empty vector.

Fig. 6. *ADTRP* regulates cell cycle progression. (A) Flow cytometry data showing decreased cell number at the S phase of cell cycle in the *ADTRP* siRNA treated HepG2 cells compared with the NC siRNA treated cells. (B) Flow cytometry data showing increased cell number at the S phase of cell cycle in HepG2 cells transfected with an *ADTRP* expression plasmid compared with cells transfected with an empty vector. (C) Graph summarizing the data from (A) and (B). (D) Flow cytometry data for *ADTRP* siRNA vs. NC siRNA in EAhy926 cells. (E) Flow cytometry data for *ADTRP* overexpression (transfection with pCDNA3.1(-)-ADTRP) vs. negative control (transfection with empty vector pCDNA3.1(-)) in EAhy926 cells. (F) Graph summarizing the data from (D) and (E). ***: P<0.001.

Fig. 7. *ADTRP* regulates cell proliferation. HepG2 or EAhy926 cells were transfected with *ADTRP* siRNA vs. NC siRNA (A, B) or *ADTRP* overexpression plasmid pCDNA3.1(-)-ADTRP vs. control (empty vector pCDNA3.1(-)) (C, D), cultured for 24 hr, 48 hr and 72 hr, and used for measuring the number of living cells using the CCK8 kit. (A)
Cell proliferation analysis for ADTRP siRNA vs. NC siRNA in HepG2 cells.  (B) Cell proliferation analysis for HepG2 cells transfected with pCDNA3.1(-)-ADTRP vs. cells transfected with empty vector pCDNA3.1(-).  (C) Cell proliferation analysis for ADTRP siRNA vs. NC siRNA in EAhy926 cells.  (D) Cell proliferation analysis for EAhy926 cells transfected with pCDNA3.1(-)-ADTRP vs. cells transfected with empty vector pCDNA3.1(-).

***: $P<0.001$; **: $P<0.01$; *: $P<0.05$.

Fig. 8. Effect of knockdown or over-expression of ADTRP on regulation of apoptosis genes by real time RT–PCR analysis.  (A) Expression of CASP7 and PDCD2 was significantly up-regulated by ADTRP knockdown in HepG2 cells.  (B) Expression of CASP7 and PDCD2 was significantly down-regulated by ADTRP overexpression in HepG2 cells.  (C) Expression of CASP7 and PDCD2 was significantly up-regulated by ADTRP knockdown in EAhy926 cells.  (D) Expression of CASP7 and PDCD2 was significantly down-regulated by ADTRP overexpression in EAhy926 cells.  ***: $P<0.001$; **: $P<0.01$.

Fig. 9. ADTRP regulates apoptosis.  HepG2 or EAhy926 cells were transfected with ADTRP siRNA vs. NC siRNA (A, B, C) or ADTRP overexpression plasmid pCDNA3.1(-)-ADTRP vs. control (empty vector pCDNA3.1(-)) (D, E, F) and used for apoptosis assays.  (A) Apoptosis analysis for ADTRP siRNA vs. NC siRNA in HepG2 cells.  (B) Apoptosis analysis for HepG2 cells transfected with pCDNA3.1(-)-ADTRP vs. cells transfected with empty vector pCDNA3.1(-).  (C) Graph summarizing apoptosis data from (A) and (B).  (D) Apoptosis analysis for ADTRP siRNA vs. NC siRNA in EAhy926 cells.  (E) Apoptosis analysis for EAhy926 cells transfected with pCDNA3.1(-)-ADTRP vs. cells transfected with empty...
vector pCDNA3.1(-). (F) Graph summarizing apoptosis data from (D) and (E). ***.

$P<0.001$. 
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*Gene set ID in ConceptGen Database; Biological knowledge type, including MeSH (Medical literature derived gene sets), MiMi (protein-centered interactions), Pfam (protein families), GO Biological Processed and Cellular Components; Number of genes in given gene set; Number of top targeted genes overlapping with given gene set; Observed P value calculated by enrichment test built-in ConceptGen; Adjusted P value for multiple testing using FDR correction.*
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