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LncRNA ENST00000602558.1 regulates ABCG1 expression and cholesterol efflux from vascular smooth muscle cells through a p65-dependent pathway

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HIGHLIGHTS

- ENST00000602558.1 specifically regulated ABCG1 expression in VSMCs.
- ENST00000602558.1 regulated ABCG1-mediated cholesterol efflux to HDL from VSMCs, but not ABCA1-mediated cholesterol efflux to apoA1.
- ENST00000602558.1 regulated ABCG1 expression and ABCG1-mediated cholesterol efflux from VSMCs through binding to p65.

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ABSTRACT

Background and aims: Long non-coding RNAs (lncRNAs) have proven to be involved in the progression of atherosclerosis and dyslipidemia. In addition, vascular smooth muscle cells (VSMCs) phenotype switching, including VSMCs-derived foam cells formation, plays a key role in the pathogenesis of atherosclerosis. LncRNA ENST00000602558.1, one of the differentially expressed lncRNAs between coronary artery disease (CAD) patients and healthy controls identified by our previous study, was located to TG and HDL susceptibility loci, but its role and underlying mechanism in the pathogenesis of atherosclerosis remain unclear. The present study aims to explore the role and underlying mechanism of ENST00000602558.1 in the regulation of cholesterol efflux from VSMCs.

Methods: ABCG1 mRNA and protein expression in VSMCs was detected using qRT-PCR and Western blot, respectively. ABCG1-mediated cholesterol efflux to HDL from VSMCs was measured by means of NBD-cholesterol fluorescence intensity. The binding of ENST00000602558.1 to p65 and p65 to *ABCG1* promoter region was detected by RNA immunoprecipitation (RIP) assay and chromatin immunoprecipitation (ChIP) assay, respectively.

Results: Overexpression of ENST00000602558.1 downregulated ABCG1 mRNA and protein expression, while knockdown of ENST00000602558.1 upregulated ABCG1 mRNA and protein expression. Consistently, ENST00000602558.1 overexpression decreased ABCG1-mediated cholesterol efflux to HDL from VSMCs by 30.38% ($p < 0.001$), and knockdown of ENST00000602558.1 increased ABCG1-mediated cholesterol efflux to HDL from VSMCs by 30.41% ($p = 0.001$). In addition to cholesterol efflux, overexpression of ENST00000602558.1 increased lipid accumulation and TC/TG levels, while knockdown of ENST00000602558.1 decreased lipid accumulation and TC/TG levels in VSMCs. Furthermore, we confirmed that ENST00000602558.1 regulated ABCG1 expression and ABCG1-mediated cholesterol efflux from VSMCs through binding to p65.

Conclusions: In conclusion, ENST00000602558.1 played an important role in mediating cholesterol efflux to HDL from VSMCs by regulating ABCG1 expression through binding to p65.

Abbreviations: ABC, ATP-binding cassette; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; acLDL, acetyl low density lipoprotein; apoA1, apolipoprotein A1; EEPD1, endonuclease–exonuclease–phosphatase family domain containing 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HDL, high-density lipoprotein; NBD-cholesterol, 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23, 24-bisnor-5-cholen-3 beta-ol; PPARG, peroxisome proliferator-activated receptor-γ; RAGE, the receptor for advanced glycation end products; SMCGS, smooth muscle cell growth supplement; SR, scavenger receptors; TRAK2, trafficking protein, kinesin binding 2

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1. Introduction

Atherosclerosis, which is the primary pathophysiologic mechanism for the development of coronary artery disease (CAD), is characterized by the deposition of cholesterol-rich plaques in the arteries [1]. Dyslipidemia is one of the key contributing factors to atherosclerosis [2]. The progression of atherosclerosis involves a complex cellular network including recruitment of monocytes, macrophages chemotaxis, proliferation, differentiation and activation, endothelial dysfunctional, vascular smooth muscle cells (VSMCs) phenotype switching, etc. [3–5]. It is generally accepted that macrophages are regarded as the prominent driving force of atherogenesis [6]. Intimal macrophages, differentiated from monocytes, phagocytize oxidized low-density lipoproteins (oxLDL) with the help of several scavenger receptors (SR) [7]. Disruption of lipid homoeostasis, including increased uptake of oxLDL and/or reduced cholesterol efflux, leads to esterified cholesterol deposition and formation of foam cells, a hallmark of the initial stages of atherosclerosis [8]. However, about half of the cells in a plaque, which appears to be macrophages, may be derived from a VSMCs lineage [9]. Cholesterol accumulation in VSMCs leads to phenotypic switching to macrophage-like cells by activating multiple proinflammatory genes, downregulating expression of VSMC marker genes and activating macrophage markers [9]. Therefore, cholesterol homeostasis breakdown in VSMCs is considered as a key factor in the development of atherogenesis events.

ATP-binding cassette A1 and G1 (ABCA1/G1), members of the ATP-binding cassette (ABC) transporter superfamily, are involved in cellular cholesterol homeostasis maintenance [10,11]. Apolipoprotein A1 (apoA1) and high-density lipoprotein (HDL)-mediated cholesterol efflux from peripheral cells are the major steps in reverse cholesterol transport process contributing to the amelioration of atherogenesis [12,13]. However, apoA1 and HDL mediate the cholesterol efflux via different mechanisms. The cholesterol efflux to apoA1 is mediated by ABCA1 through two distinct mechanisms, including formation of cholesterol-apoA1 complexes and binding between apoA1 and ABCA1 on the cell surface before internalization, while the cholesterol efflux to HDL is mediated by ABCG1, which promotes changes of plasma membrane phospholipids organization or helps sterol molecules entry into the hydrophilic water layer [14–17]. Besides, as two major pathways in the release of cholesterol from cells, approximately 70% of the efflux is attributable to ABCA1 and ABCG1 in cholesterol-enriched cells [18]. Therefore, deficiency of ABCA1 and/or ABCG1 leads to cellular cholesterol accumulation and atherosclerosis *in vivo* [11,19]. The nuclear factor-κB (NF-κB) family regulates a variety of biological processes as transcription factors. According to bioinformatics predictions, *ABCG1* promoter region containing potential binding sites of RELA (p65) [20,21]. Furthermore, NF-κB activation decreased the level of ABCG1, while the specific inhibitor of NF-κB reversed the NF-κB-induced suppression of ABCG1 expression [22] and thus, p65 might play an important role in the regulation of ABCG1 expression.

Long noncoding RNAs (lncRNAs), an important category of non-coding transcripts of more than 200 nucleotides in length, not only regulate gene expression in physiological condition but also account for the pathogenesis of cardiovascular disease, for instance, inflammation, endothelial function, atheromatous plaques formation, diabetes and dyslipidemia [23,24]. Our previous study systematically identified the differentially expressed lncRNAs between patients with CAD and healthy controls by profiling transcriptome-wide lncRNAs expression [25]. ENST00000602558.1, one of the differentially expressed lncRNAs, is located on a CAD [26–28], triglyceride (TG) [29–31] and HDL [29,30] susceptibility region (chr12q24.31). Therefore, it is reasonable that ENST00000602558.1 might be involved in the pathogenesis of atherosclerosis through mediating cholesterol homeostasis. In the present study, we explore the effect of ENST00000602558.1 on ABCG1 expression and ABCG1-mediated cholesterol efflux to HDL from VSMCs, and potential molecular mechanism involved in cholesterol efflux regulation by ENST00000602558.1.

2. Materials and methods

2.1. Cell culture, transfection and infection

Human Umbilical Vein Smooth Muscle Cells (ScienCell, Catalog #8020, San Diego, CA, USA) were cultured in Smooth Muscle Cell Medium containing 2% fetal bovine serum (FBS, Cat. No. 0010), 1% smooth muscle cell growth supplement (SMCGS, Cat. No. 1152) and 1% penicillin/streptomycin solution (P/S, Cat. No. 0503). Cell cultures were maintained in a humidified 5% CO2 incubator at 37 °C, and the medium was replaced every 72 h. Cells were subcultured at a ratio of 1:3 with 0.25% trypsin-EDTA (gibco, Life Technology, USA) when cells were cultured to subconfluence. VSMCs were used from passages 4 to 6 in the following experiments. VSMCs were seeded at a density of 1.0×10^5 cells per well in a 12-well plate 24 h prior to transfection. VSMCs were transfected with 50 nM of siRNAs against ENST00000602558.1 (si-ENST00000602558.1, Ribobio, Guangzhou, China) or p65 (si-p65, GenePharma, Shanghai, China) and corresponding negative control siRNAs (si-NC, GenePharma, Shanghai, China) for 48 h using Lipofectamine$^{\mathrm{TM}}$RNAiMax (Invitrogen, USA) according to the manufacturer's protocol. For adenovirus-mediated overexpression of ENST00000602558.1, a recombinant adenoviral vector containing full-length cDNA of ENST00000602558.1 (Ad-ENST00000602558.1) (Vigenebio, Shandong, China) was added into the culture medium at 100 multiplicity of infection (MOI) for 48 h. Recombinant adenovirus (Ad-Null) was used as the negative control. The VSMCs which had been transfected or infected for 24 h were exposed to 50 μg/ml acetyl low density lipoprotein (acLDL) (Yiyuan Biotech, Guangzhou, China) for additional 24 h.

2.2. RNA extraction and quantitative real-time PCR

Total cellular RNA was extracted by TRIzol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. RNA quantity was assessed using NanoDrop 2000 (NanoDrop Products, Wilmington, DE, USA). Reverse transcription was carried out with 1 μg total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) followed by fluorescent quantitative real-time polymerase chain reaction (qRT-PCR) using SYBR Green detection chemistry with an ABI ViiA™ 7 system. Specific primers for the genes detected are listed in Supplementary Table 1. The relative fold-change was determined using the double delta method ($2^{-\triangle\triangle Ct}$) normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level.

2.3. Western blot analysis

Cellular total proteins were extracted. An equal amount of total proteins was loaded onto 10% SDS-PAGE and transferred to a nitrocellulose membrane via the Bio-Rad transfer blotting system. The binding was blocked with 5% skim milk (Difco, Cat. No. 232100, USA) in 1 × TBST (Applygen Technologies Inc., Beijing, China) for 1 h at room temperature. Blots were incubated with anti-ABCA1 (1:500, abcam, ab18180), anti-ABCG1 (1:2000, abcam, ab52617), anti-p65 (1:500, Cell Signaling Technology Inc., L8F6, Massachusetts, USA) or anti-GAPDH (1:4000, abcam, ab181602) antibodies overnight at 4 °C. After washing with 1 × TBST, the blots were incubated with horseradish peroxidase-conjugated anti-rabbit or anti-rat secondary antibody (1:3000, ZSGB-BIO, Beijing, China) for 1 h at room temperature. Finally, immunoreactive bands were detected by enhanced chemiluminescent system (ProteinSimple, Santa Clara, CA, USA).

2.4. Oil Red O staining

Oil red O staining was conducted using Oil Red O stain kit (Solarbio Science & Technology Co., Ltd, Beijing, China) according to the manufacturer's protocol. The cells covered by distilled water were observed by Leica DM IL LED microscope (Leica Microsystems Pty Ltd, USA).

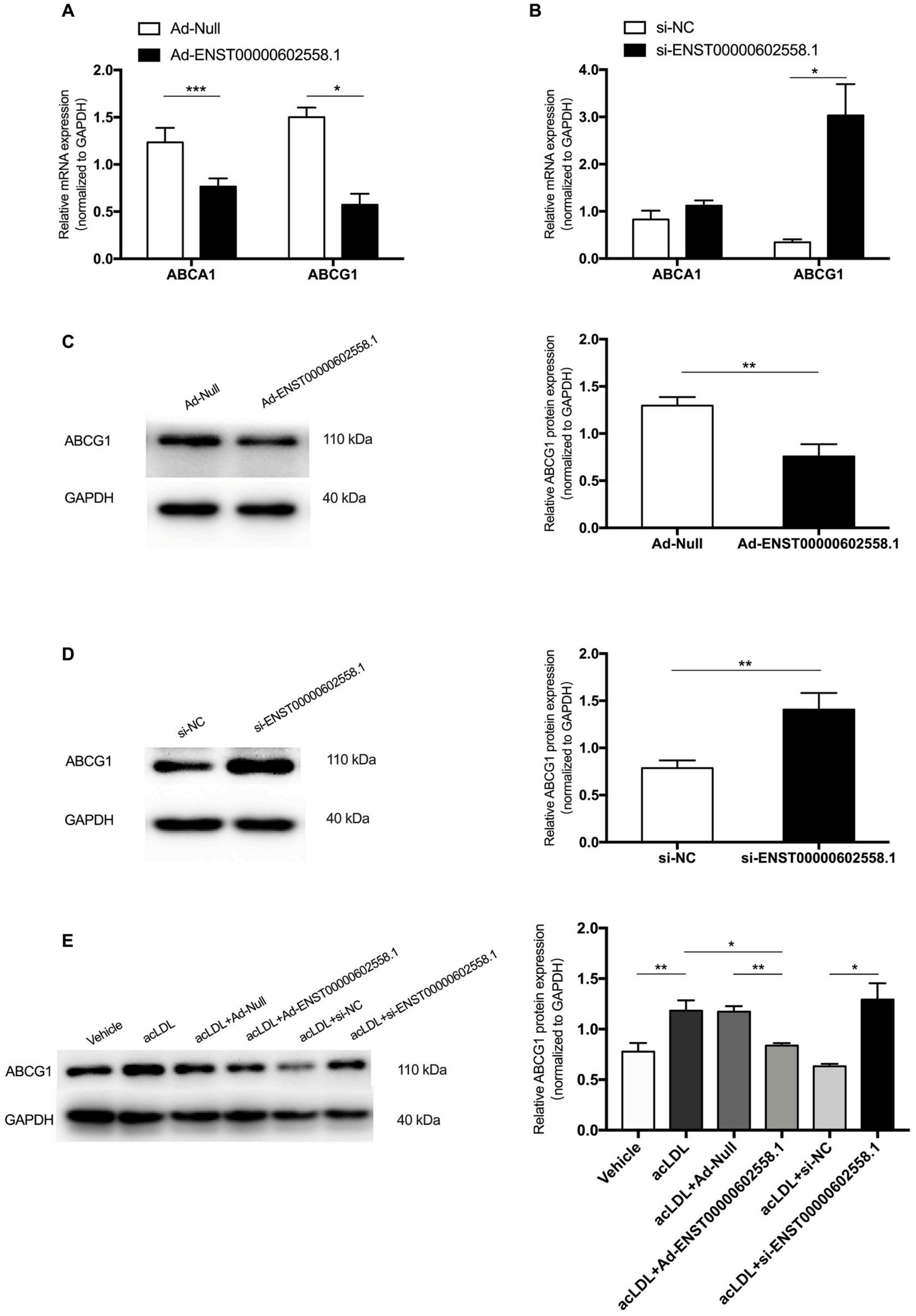

Fig. 1. ENST00000602558.1 regulates *ABCG1* gene expression in human VSMCs.

(A) Effect of ENST00000602558.1 overexpression on mRNA expression of *ABCA1* and *ABCG1* genes. (B) Effect of ENST00000602558.1 knockdown on mRNA expression of *ABCA1* and *ABCG1* genes. (C) Effect of ENST00000602558.1 overexpression on ABCG1 protein expression. (D) Effect of ENST00000602558.1 knockdown on ABCG1 protein expression. (E) Regulation of ABCG1 protein expression by ENST00000602558.1 in VSMCs in the presence of acLDL (50 μg/ml). Data are presented as mean ± standard error. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* the control group. Each experiment was performed three times.

2.5. Cellular total cholesterol and triglyceride assay

Cellular total cholesterol (TC) and triglyceride (TG) were tested by enzymatic assay using cholesterol assay kits (Applygen Technologies, Beijing, China) and TG assay kit (Applygen Technologies, Beijing, China) in accordance with the manufacturer's protocol. The absorbance values were detected by Infinite M200 Pro (TECAN, Switzerland) using 550 nm filters in 96-well plates.

2.6. Cholesterol efflux assay

To assess cholesterol efflux, VSMCs were exposed to 5 μmol/L 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23, 24-bisnor-5-cholen-3 beta-ol (NBD-cholesterol) (Molecular Probes, Eugene, OR, USA) in serum-free medium for 4 h at 37 °C with or without 24 h-pretreatment of 50 μg/ml acLDL (Yiyuan Biotech, Guangzhou, China) and then washed with phosphate buffered saline (PBS) (HyClone, Logan, UT) twice. The VSMCs were treated with siRNA or adenovirus followed by incubation with 50 μg/ml human HDL (Yiyuan Biotech, Guangzhou, China) or 15 μg/ml lipid-free human apoA1 (Sigma Aldrich, St. Louis, MO, USA) in medium containing 0.2% bovine serum albumin (BSA) (Solarbio, Cat#A8020, Beijing, China) for 4 h. Following incubation, the medium was collected and the cellular cholesterol was extracted using 0.1% Triton X-100 (Gibco Life Technologies, Carlsbad, CA, USA) for the fluorescence intensity (FI) detection. The FI of NBD-cholesterol in the medium and cell lysate was detected by Infinite M200 Pro (TECAN, Switzerland) using 469 nm excitation and 537 nm emission filters in a black polystyrene 96-well plate (Costar, Corning Incorporated, USA). The cholesterol efflux was expressed as dividing the FI in the medium by the sum of the FI in the medium and cell lysate.

2.7. RNA immunoprecipitation (RIP) assay

EZ-Magna RIP kit (Millipore, Billerica, MA, USA) was used to perform RIP assay. A total of 1.0×10^7 of VSMCs were collected and lysed in RIP lysis buffer, followed by the incubation of protein A/G magnetic beads and IgG antibody (Millipore, Billerica, MA, USA) or p65 antibody (abcam, ab19870). RNA was eluted from magnetic beads-binding complexes according to the manufacturer's instructions. Finally, qRT-PCR assay was performed to detect the enrichment patterns of ENST00000602558.1 by antibody against IgG or p65.

2.8. Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation (ChIP) assays were performed by using Agarose ChIP Kit (26156, Pierce Protein Biology, USA) as described in the manufacturer's instructions. Cells were crosslinked with 1% paraformaldehyde solution for 10 min, followed by the addition of glycine for 5 min. Crosslinked cells then were lysed by the lysis buffer containing protease inhibitors. To obtain DNA fragments with an average length of 300 base pairs (bps), 0.25 μl micrococcal nuclease was added and incubated for 5 min. Normal IgG were used as the negative control, and anti-p65 (abcam, ab19870) were used for immunoprecipitation. After DNA recovery, purified DNA was subjected to qRT-PCR analysis. The amount of immunoprecipitated DNA was normalized to the input DNA.

2.9. Statistical analysis

Values were expressed as mean ± standard deviation (SD). All data were from at least three independent experiments, each performed in triplicate. Data were analyzed by Student's *t*-test or by ANOVA. $p < 0.05$ was considered statistically significant. All data were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. ENST00000602558.1 specifically regulates ABCG1 expression in VSMCs

To determine whether ENST00000602558.1 regulates endogenous levels of ATP-binding cassette transporter family members in VSMCs, we detected mRNA and protein levels of *ABCA1* and *ABCG1* in VSMCs infected with adenovirus (2.0×10^7 pfu/ml) or transfected with si-ENST00000602558.1 (50 nM) for 48 h. As shown in Fig. 1A and B, the overexpression of ENST00000602558.1 significantly downregulated *ABCA1* and *ABCG1* mRNA expressions, while knockdown of ENST00000602558.1 only upregulated *ABCG1* mRNA expression. There were no significant differences in *ABCA1* mRNA expression in VSMCs transfected with si-ENST00000602558.1 compared with control cells (Fig. 1B). Corresponding to mRNA level, the overexpression of ENST00000602558.1 downregulated ABCG1 protein level (Fig. 1C), while knockdown of ENST00000602558.1 upregulated ABCG1 protein level (Fig. 1D). However, both the overexpression and knockdown of ENST00000602558.1 resulted in lower ABCA1 protein level (Supplementary Fig. 1A and B). These results demonstrated that ENST00000602558.1 specifically regulated ABCG1 expression, but not ABCA1 expression in VSMCs.

We further determined the regulation of ABCG1 expression by ENST00000602558.1 in lipid-loaded VSMCs. As shown in Fig. 1E, ABCG1 protein expression was increased in acLDL-treated VSMCs, and the overexpression of ENST00000602558.1 significantly downregulated ABCG1 protein level, while knockdown of ENST00000602558.1 upregulated ABCG1 protein level in the presence of acLDL. These results demonstrated that ENST00000602558.1 regulated ABCG1 expression in VSMCs not only under basal condition but also in the presence of acLDL.

To further determine the cell type-specific regulation of *ABCA1* and *ABCG1* genes by ENST00000602558.1, we detected mRNA and protein levels of ABCA1 and ABCG1 in THP-1-derived macrophages transfected with si-ENST00000602558.1 (50 nM) for 48 h. Interestingly, knockdown of ENST00000602558.1 only slightly upregulated *ABCG1* mRNA expression rather than *ABCA1* (Supplementary Fig. 2A) and no significant differences in ABCG1 protein expression could be found in THP-1-derived macrophages transfected with si-ENST00000602558.1 compared with control cells (Supplementary Fig. 2B), indicating cell type-specific regulation of ABCG1 by ENST00000602558.1.

Additionally, mRNA expression of genes involved in cholesterol influx (*CD36*, *MSR1*, *OLR1*) and esterification (*ACAT1*, *NCEH*) was detected (Supplementary Fig. 3). The overexpression of ENST00000602558.1 significantly upregulated *CD36, MSR1, OLR1*, but not *NCEH* mRNA expression, and downregulated *ACAT1* mRNA expression, while knockdown of ENST00000602558.1 only downregulated *NCEH* mRNA expression. Therefore, these genes involved in cholesterol influx and esterification were not specifically regulated by ENST00000602558.1 in VSMCs, indicating that ENST00000602558.1 might not regulate cholesterol influx and esterification in VSMCs.

Taken together, ENST00000602558.1 specifically regulated ABCG1 expression in VSMCs.

3.2. ENST00000602558.1 regulates cholesterol efflux to HDL from VSMCs

Since ABCG1 expression was regulated by ENST00000602558.1, we explored the effect of ENST00000602558.1 on ABCG1-mediated cholesterol efflux to HDL from VSMCs. As shown in Fig. 2A and B, ABCG1-mediated cholesterol efflux to HDL from VSMCs was decreased by 30.38% in VSMCs by overexpression of ENST00000602558.1. In agreement, knockdown of ENST00000602558.1 in VSMCs increased ABCG1-mediated cholesterol efflux to HDL by 30.41%. However, the overexpression or knockdown of ENST00000602558.1 did not alter ABCA1-mediated cholesterol efflux to apoA1 in VSMCs (Supplementary

Fig. 4A and B). Taken together, these findings confirmed that ENST00000602558.1 regulated ABCG1-mediated cholesterol efflux to HDL from VSMCs, but not ABCA1-mediated cholesterol efflux to apoA1.

Furthermore, ABCG1-mediated cholesterol efflux to HDL from VSMCs in the presence of acLDL was detected. As shown in Fig. 2C, ABCG1-mediated cholesterol efflux to HDL from VSMCs was increased in the presence of acLDL, which was consistent with previous studies [32,33]. Interestingly, overexpression of ENST00000602558.1 decreased ABCG1-mediated cholesterol efflux to HDL by 65.2% in VSMCs treated with acLDL, which was more remarkable than under basal conditions. Knockdown of ENST00000602558.1 increased ABCG1-mediated cholesterol efflux to HDL in VSMCs treated with acLDL by 13.3%. Thus, ENST00000602558.1 regulated ABCG1-mediated cholesterol efflux to HDL in VSMCs both under basal conditions and in the presence of acLDL.

In addition to cholesterol efflux, we also examined the effect of ENST00000602558.1 on lipid accumulation and cholesterol levels in VSMCs. As shown in Fig. 2D, overexpression of ENST00000602558.1 drastically increased lipid accumulation in VSMCs, while knockdown of ENST00000602558.1 markedly attenuated lipid accumulation in VSMCs. Consistent with the results above, cellular TC and TG levels were significantly increased in VSMCs infected with Ad-ENST00000602558.1 compared with control cells (Fig. 2E), while TC and TG levels were decreased in VSMCs transfected with si-ENST00000602558.1 (Fig. 2F).

Thus, overexpression of ENST00000602558.1 promoted lipid accumulation and TC/TG levels in VSMCs along with downregulation of ABCG1-mediated cholesterol efflux to HDL, and knockdown of ENST00000602558.1 attenuated lipid loading and TC/TG levels in VSMCs along with the upregulation of ABCG1-mediated cholesterol efflux to HDL.

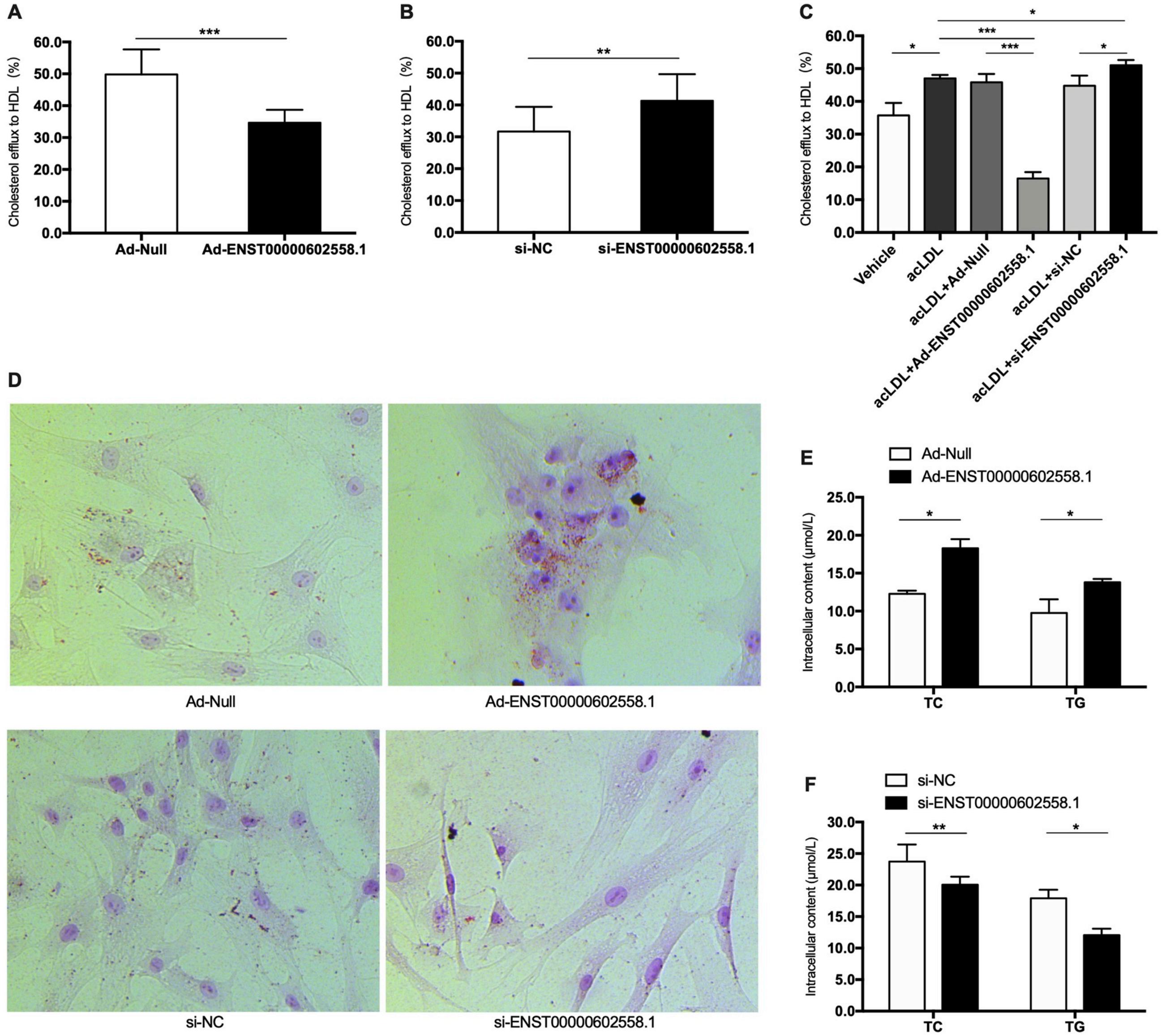

Fig. 2. ENST00000602558.1 regulates lipid accumulation, TC/TG contents and cholesterol efflux in VSMCs.
(A) Effect of ENST00000602558.1 overexpression on ABCG1-mediated cholesterol efflux to HDL. (B) Effect of ENST00000602558.1 knockdown on ABCG1-mediated cholesterol efflux to HDL. (C) The regulation of ABCG1-mediated cholesterol efflux to HDL in VSMCs by ENST00000602558.1 in the presence of acLDL (50 μg/ml). (D) Regulation of intracellular lipid accumulation by ENST00000602558.1. detected by Oil Red O staining. Images are representative of three separate experiments (magnification: ×200). (E) Effect of ENST00000602558.1 overexpression on cellular TC and TG contents. (F) Effect of ENST00000602558.1 knockdown on cellular TC and TG contents. Data are presented as mean ± standard error. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs.* the control group. Each experiment was performed three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Regulation of ABCG1 expression and ABCG1-mediated cholesterol efflux to HDL by ENST00000602558.1 in VSMCs is a p65 dependent pathway

Previous studies indicated that NF-κB inhibitor led to an increase in HDL-mediated cholesterol efflux by upregulating ABCG1 expression in VSMCs [34]. Therefore, we detected the mRNA and protein levels of ABCG1 in VSMCs transfected with si-p65 (50 nM) or si-NC. Our results showed that knockdown of p65 increased both mRNA and protein expression of the *ABCG1* gene (Fig. 3A and B). Although *ABCA1* mRNA was increased by knockdown of p65 (Fig. 3A), ABCA1 protein was not significantly increased by knockdown of p65 (Fig. 3B). We further investigated whether p65 was involved in ABCG1-mediated cholesterol efflux to HDL from VSMCs. As expected, knockdown of p65 led to an increase of cholesterol efflux to HDL from VSMCs by 12.15% (Fig. 4A), while cholesterol efflux to apoA1 was not significantly increased (Supplementary Fig. 5), indicating that p65 specifically regulated ABCG1-mediated cholesterol efflux to HDL from VSMCs. Therefore, we speculated that p65 might be involved in the regulation of ABCG1 expression and further ABCG1-mediated cholesterol efflux to HDL by ENST00000602558.1. As shown in Fig. 4B, ENST00000602558.1 overexpression significantly downregulated ABCG1 protein level in the Ad-ENST00000602558.1 + si-NC group compared with the Ad-Null + si-NC group, and downregulation of ABCG1 protein was remarkably reversed by knockdown of p65 in the Ad-ENST00000602558.1 + si-p65 group compared with the Ad-ENST00000602558.1 + si-NC group. Similarly, ABCG1-mediated cholesterol efflux was decreased in the Ad-ENST00000602558.1 + si-NC group compared with the Ad-Null + si-NC group, which was also moderately but significantly reversed by knockdown of p65 in the Ad-ENST00000602558.1 + si-p65 group compared with the Ad-ENST00000602558.1 + si-NC group (Fig. 4C). These results demonstrated that regulation of ABCG1 expression and further ABCG1-mediated cholesterol efflux to HDL by ENST00000602558 was a p65-dependent pathway in VSMCs.

3.4. The direct biding of ENST00000602558.1 to p65 inhibited ABCG1 transcription

Before performing RIP assay, RNA-protein interactions prediction was conducted using RNA-Protein Interaction Prediction (RPISeq) (http://pridb.gdcb.iastate.edu/RPISeq/index.html) based on the Protein-RNA Interface Database (PRIDB) [35,36]. Interaction probabilities generated by RPISeq were presented as two variants: RPISeq-SVM, which uses a Support Vector Machine (SVM) classifier, and RPISeq-RF, which uses a Random Forest classifier. The outputs of RPISeq are interaction propensities for specific RNA-protein pair using 0.5 as the “positive” probability threshold [35]. Both variants were more than 0.5 (RPISeq-SVM = 0.99; RPISeq-RF = 0.55) for the prediction, indicating that ENST00000602558.1 might bind to p65 specifically.

RIP assay was performed using p65 antibody to further decipher the direct binding between ENST00000602558.1 and p65. The results showed that ENST00000602558.1 was substantially enriched by p65 antibody compared with IgG antibody (Fig. 5A), indicating the direct binding of ENST00000602558.1 to p65. Furthermore, ChIP assays were

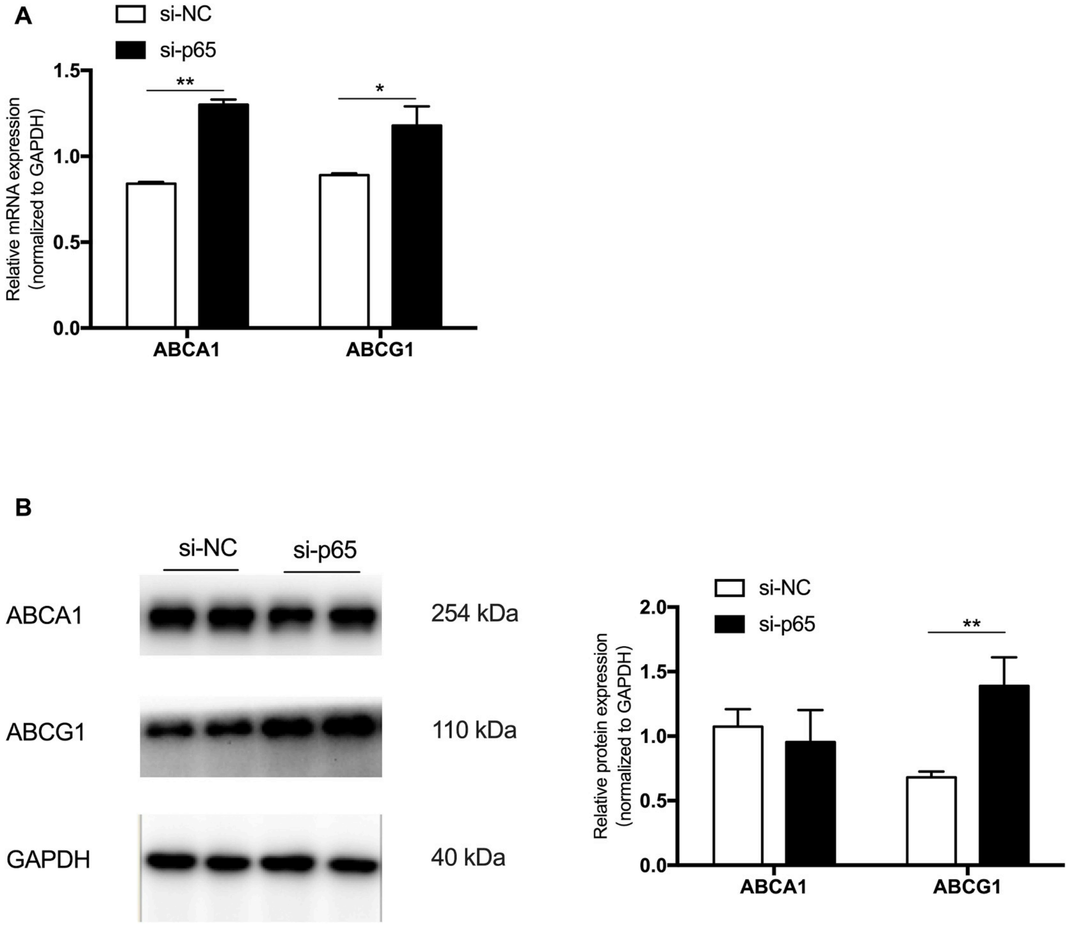

Fig. 3. Knockdown of p65 increased ABCG1 expression in human VSMCs.
(A) Effect of p65 knockdown on mRNA expression of *ABCA1* and *ABCG1* genes. (B) Effect of p65 knockdown on protein expression of *ABCA1* and *ABCG1* genes. Data are presented as mean ± standard error. $^*p < 0.05$, $^{**}p < 0.01$ *vs*. the control group. Each experiment was performed three times.

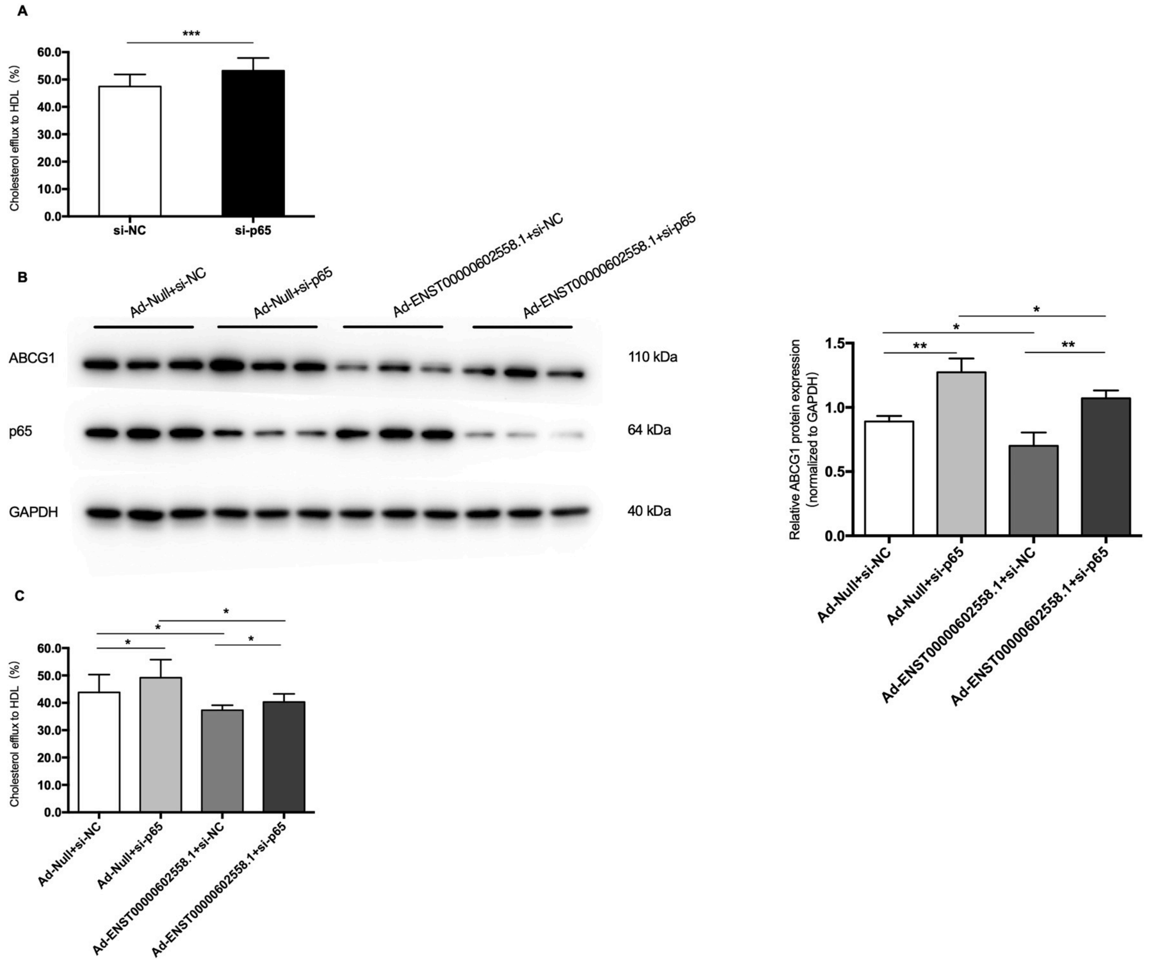

Fig. 4. Regulation of ABCG1 expression and ABCG1-mediated cholesterol efflux to HDL by ENST00000602558.1 in VSMCs was a p65 dependent pathway.
(A) Effect of p65 knockdown on ABCG1-mediated cholesterol efflux to HDL from VSMCs. (B) Reverse effect of p65 knockdown on ABCG1 protein expression inhibited by the overexpression of ENST00000602558.1. (C) Rescue effect of p65 knockdown on ABCG1-medicated cholesterol efflux to HDL from VSMCs inhibited by the overexpression of ENST00000602558.1. Data are presented as mean ± standard error. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs.* the control group. Each experiment was performed three times.

performed to detect the recruitment of p65 to *ABCG1* promoter region. As shown in Fig. 5B, *ABCG1* promoter region was significantly enriched by p65 antibody compared with IgG antibody, indicating the recruitment of p65 to *ABCG1* promoter region. Taken together, ENST00000602558.1 regulated ABCG1 expression and ABCG1-mediated cholesterol efflux from VSMCs through binding to p65.

4. Discussion

Since vascular cholesterol overloading is a major cause of atherosclerosis, the efflux process is of great importance [37,38]. VSMCs phenotype switching plays a critical role in the pathogenesis of vascular lesion and contributes to plaque formation [39]. VSMC phenotypic switching leads to less-differentiated forms, including macrophage-like cells, which promote inflammation, monocyte recruitment and the development of atherosclerosis [39]. In addition, VSMCs can also be converted into foam cells by adopting a 'macrophage-like' phenotype and taking up lipids [39,40]. Compared with macrophages, the knowledge of cholesterol homeostasis regulation in VSMCs is limited. In the present study, we provide a novel evidence that ENST00000602558.1 regulates ABCG1 expression and ABCG1-mediated cholesterol efflux to HDL by binding to p65 in VSMCs.

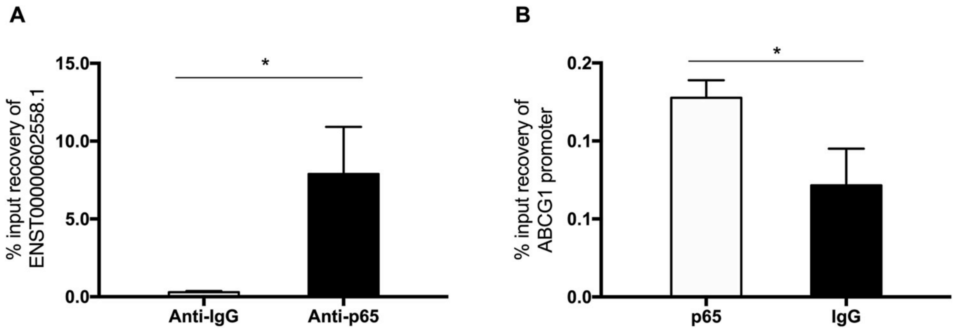

Fig. 5. The binding of ENST00000602558.1 to p65 regulate *ABCG1* transcription in VSMCs.
(A) RIP and qRT-PCR assays were performed to explore the binding efficiency between ENST00000602558.1 and p65. (B) ChIP and qRT-PCR assays were performed to detect the binding of p65 to the promoter region of *ABCG1* gene. Data are presented as mean ± standard error. *$p < 0.05$ *vs.* the control group. Each experiment was performed three times.

Functions of lncRNAs can be classified into several categories including imprinting acts to inhibit the targeted locus expression, recruiting functional proteins to direct diverse biological phenomena, interacting with promoters of protein-coding genes and sequestering miRNAs functions as molecular sponge [41]. Furthermore, lncRNAs have now been confirmed to be associated with various cardiovascular conditions and the associated risk factors including atherosclerosis, dyslipidemia and metabolic syndrome [42]. Single nucleotide variations around the locus of ENST00000602558.1, such as rs825476 [26], rs11057401 [28] and rs4765127 [30,43], were reported to be associated with type 2 diabetes, CAD, insulin resistance, TG and HDL [25]. Consequently, it is reasonable to speculate that ENST00000602558.1 may be involved in the progression of atherosclerosis or CAD, but the underlying mechanism remains unclear. Furthermore, it is well established that ABCA1 and ABCG1, the membrane lipid translocases, prevent the pathogenesis of atherosclerosis due to their transporter function in cholesterol efflux, the initial step in reverse cholesterol transport [13,44]. ABCA1 mediates the cellular efflux of cholesterol and phospholipids to apoA1 while ABCG1 mediates cholesterol efflux through interactions with HDL particles [10,45]. Zinc finger protein 202 (ZNF202) is a transcriptional repressor of *ABCA1* and *ABCG1* gene and inhibits their promoter transcriptional activities [46]. Similarly, the receptor for advanced glycation end products (RAGE) ligands suppresse *ABCA1* and *ABCG1* transcription through peroxisome proliferator-activated receptor-γ(PPARG)-responsive promoter elements [47]. Although liver X receptor (LXR) α sustains the baseline protein expression of ABCA1, ABCG1 and SR-BI, as well as HDL- and apoA1-mediated cholesterol efflux [48], endonuclease–exonuclease–phosphatase family domain containing 1 (EEPD1), a transcriptional target of LXRs, specifically regulates ABCA1 abundance and apoA1-mediated cholesterol efflux from macrophages [49]. Besides, the trafficking protein, kinesin binding 2 (TRAK2) is a specific regulator of LXR-mediated ABCA1 expression at transcription level [50]. Interestingly, our results confirmed that ENST00000602558.1 specifically regulated the expression of *ABCG1* gene but not *ABCA1* gene in VSMCs, and ABCG1-mediated cholesterol efflux to HDL both under basal condition and in the presence of acLDL, for the first time. It is well established that lncRNAs contribute to cell type-specific regulation of target genes [51]. MeXis, an LXR-responsive lncRNA, regulates ABCA1 expression and apoA1-mediated cholesterol efflux from macrophages in a cell type-specific manner [51]. However, in the present work, ENST00000602558.1 regulated *ABCG1* gene and ABCG1-mediated cholesterol efflux to HDL in VSMCs but not THP-1-derived macrophages (Supplementary Fig. 2), indicating VSMC type-specific regulation of *ABCG1* by ENST00000602558.1.

The imbalance of cholesterol influx, esterification and efflux can lead to lipid overload within cells, which contributes to the formation of foam cells and atheroma development [8]. Under atherogenic conditions, influx of cholesterol is increased due to the enhanced expression of SR such as cluster of differentiation 36 (CD36), macrophage scavenger receptor 1 (MSR1) and oxidized low-density lipoprotein receptor 1 (OLR1), while the expression of cholesterol transporters ABCA1/G1 is downregulated [52]. Despite the influx and efflux pathways, excess esterification of cholesterol due to higher acetyl-coenzyme A acetyltransferase 1 (ACAT1) level and lower neutral cholesterol ester hydrolase 1 (NCEH) level results in lipid accumulation [8,52]. The present study confirmed that ENST00000602558.1 regulated ABCG1-mediate cholesterol efflux to HDL along with the deposition of lipid and TC/TG. However, the expression of SR and esterification-related genes were not regulated by ENST00000602558.1 (Supplementary Fig. 3). Therefore, ENST00000602558.1 might promote lipid-loaded VSMCs formation by restricting cholesterol release from cytoplasm.

p65 is a member of NF-κB family, which is involved in diverse biological processes [20]. The result of ChIP assay in the present work confirmed the binding of p65 to *ABCG1* promoter, which was consistent with a previous work based on bioinformatics prediction [21]. Importantly, the inhibitor of NF-κB, such as Bay11-7085 and TPCK, led to selective transcription of *ABCG1,* with an increase of cytoplasmic I-κBα protein level and a decrease of nuclear p65 level [34]. In the present study, knockdown of p65 promoted the expression of ABCG1 and ABCG1-mediated cholesterol efflux to HDL in VSMCs, indicating a negative regulatory role of p65 for *ABCG1* gene expression and ABCG1-mediated cholesterol efflux to HDL. Therefore, we speculated that p65 was involved in the regulation of ABCG1 expression and ABCG1-mediated cholesterol efflux by ENST00000602558.1. A phenotype rescue assay confirmed that downregulation of ABCG1 expression and ABCG1-mediated cholesterol efflux by ENST00000602558.1 could be reversed by knockdown of p65, demonstrating a p65 dependent pathway. Furthermore, the binding of ENST00000602558.1 to p65 and p65 to the *ABCG1* promoter region was confirmed by RIP and ChIP assay, respectively. Thus, we speculated that binding of ENST00000602558.1 to p65 could suppress ABCG1 expression and ABCG1-mediated cholesterol efflux by enhancing the negative regulatory activity of p65 on *ABCG1* gene transcription.

In summary, we revealed a novel regulatory role of ABCG1 expression and ABCG1-mediated cholesterol efflux to HDL in VSMCs by ENST00000602558.1 through binding to p65. VSMCs play a critical role in the development of atherosclerosis, thus it is critical to identify VSMCs-associated therapeutic targets [39]. The efficiency of ENST00000602558.1 was higher in the presence of acLDL, raising the possibility of ENST00000602558.1 as a potential therapeutic agent for atherosclerosis. Nevertheless, the effect and underling mechanism of ENST00000602558.1 and p65 on the development of atherosclerosis and dyslipidemia are still in their infancy. Further research is needed to better understand the mechanism whereby ENST00000602558.1 regulates the progression of atherosclerosis before ENST00000602558.1-based therapeutics can be taken into clinical practice.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Can Cai and Laiyuan Wang designed the experiments. Can Cai performed the research, analyzed the data and wrote this manuscript. Can Cai, Huijuan Zhu, Xiaotong Ning and Lin Li performed the experiment. Bin Yang, Shufeng Chen, Xiangfeng Lu and Laiyuan Wang takes responsibility for the integrity of the data and the accuracy of the data analysis, and reviewed and edited the manuscript. Laiyuan Wang and Dongfeng Gu designed the study and supervised all the sample selection, data analysis and interpretation. All coauthors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2019.04.204.

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