Endothelial expression of human ABCA1 in mice increases plasma HDL cholesterol and reduces diet-induced atherosclerosis

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Running title: Endothelial ABCA1 overexpression reduces atherosclerosis

Abbreviations: hABCA1 – human ABCA1 transporter; EC – endothelial cells; HDL-C – HDL-cholesterol; HFHC - high fat high cholesterol; knockout – Ko; RCT – reverse cholesterol transport.
ABSTRACT

The role of endothelial ABCA1 expression in reverse cholesterol transport (RCT) was examined in transgenic mice, using the endothelial specific Tie2 promoter. Human ABCA1 was significantly expressed in endothelial cells (EC) of most tissues except the liver. Increased expression of ABCA1 was not observed in resident peritoneal macrophages. ApoA-I mediated cholesterol efflux from aortic EC was 2.6-fold higher ($P<0.0001$) for cells from transgenic versus control mice. On normal chow diet, Tie2hABCA1 transgenic mice had a 25% ($P<0.0001$) increase in HDL-cholesterol (HDL-C) and more than a 2-fold increase of eNOS mRNA in the aorta ($P<0.04$). After 6 months on a high-fat, high-cholesterol (HFHC) diet, transgenic mice compared to controls had a 40% increase in plasma HDL-C ($P<0.003$) and close to 40% decrease in aortic lesions ($P<0.02$). Aortas from HFHC fed transgenic mice also showed gene expression changes consistent with decreased inflammation and apoptosis. Beneficial effects of the ABCA1 transgene on HDL-C levels or on atherosclerosis were absent when the transgene was transferred onto ApoE or Abca1 knockout mice. In summary, expression of hABCA1 in EC appears to play a role in decreasing diet-induced atherosclerosis in mice and is associated with increased plasma HDL-C levels and beneficial gene expression changes in EC.

Supplementary key words: endothelial cells; cholesterol efflux; reverse cholesterol transport.
INTRODUCTION

Endothelial cells (EC) account for only 1-2% of all cells in the body, but in the average size adult, EC cover a surface area over 1000 m² and weigh approximately 1 kg (1, 2). Because of their role as a barrier to the plasma compartment, they are known to modulate atherogenesis in multiple ways (3, 4). When inflamed and affected by factors, such as hypercholesterolemia, hypertension, and type 2 diabetes, EC express several adhesion proteins (5). Monocytes and T-cells attach to these adhesion proteins and gain entry into subendothelial space, where they initiate the development of atherosclerosis. EC also express several scavenger receptors, including CD36 and Lox-1, which mediate the uptake of oxidized LDL and promote atherosclerosis (6). The EC surface is also the site of action of lipoprotein lipase, hepatic lipase and endothelial lipase, which promote the lipolysis of lipoproteins and facilitate the transfer of lipids across the vessel wall (3, 7).

Reverse cholesterol transport (RCT) is the pathway by which excess cellular cholesterol is removed by HDL from peripheral cells and transported to the liver for excretion (8, 9). Based on various transgenic and Ko mouse models (10-14), ABCA1 is known to play a key role in the biogenesis of HDL. Approximately 70% of nascent HDL is made in the liver when apoA-I, which is also produced by hepatocytes, interacts with hepatic ABCA1 transporters (13, 14). The only other tissue that synthesizes significant amounts of apoA-I and also expresses ABCA1 is the intestine, which is thought to be responsible for most of the remainder of nascent HDL formation (14). The lipidation by ABCA1 of apoA-I released from lipoproteins, during remodeling by lipases and other enzymes and lipid transfer proteins, also contributes to the maintenance of plasma HDL-cholesterol (HDL-C) (15).

EC express several genes involved in RCT, such as ABCA1, ABCG1 and SR-B1 (16-18), which are key transporters and receptors that are known to promote either the cellular efflux or uptake of cholesterol to and from HDL. Endothelial expression of ABCG1 was recently shown to protect against endothelial dysfunction in mice fed high fat high cholesterol (HFHC) diet (19). It is also has been shown that ABCA1 participates in the transport of lipid-poor apoA-I across EC, whereas ABCG1 and SR-B1 facilitate
the transcytosis of HDL across endothelium (18, 20, 21). In order to further examine the role of ABCA1 in EC, we describe the development of transgenic mice overexpressing human ABCA1 (hABCA1), using the endothelial specific Tie2 promoter (22). Endothelial specific expression of hABCA1 was found to raise plasma HDL-C and reduce diet-induced atherosclerosis.
MATERIALS AND METHODS

Generation of Tie2hABCA1 transgenic mice

Full-length (6.88-kb) hABCA1 cDNA was flanked by Not I linkers and inserted into a unique Not I cloning site of the expression vector pSPTg.T2FpAXK (22, 23). The vector has a 2.1 kb mouse Tie2 promoter, a SV40 3’ terminal untranslated sequence with a polyA signal and a 1.6 kb Tie2 enhancer, which results in specific transgene expression in EC of mice (22, 23). The plasmid was digested with Sal I, and a 10.7-kb DNA fragment, containing the complete expression cassette, including the Tie2 promoter, was isolated from a 0.8% agarose gel and purified by CsCl density gradient ultracentrifugation (Beckman TL-100 table top ultracentrifuge at 95000 RPM for 24 hrs at 20ºC). After dialysis in 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, the DNA fragment was microinjected into pronuclei of fertilized eggs from C57Bl/6J females (Jackson Laboratory, ME, USA).

For genotyping, DNA was isolated from tail clips or blood, with the Maxwell16 System (Promega) and analyzed by real-time quantitative PCR, with ABI 7900HT and TaqMan expression assays (see Supplementary Table 1). Tie2hABCA1 DNA was used for the calibration curve. A strain of mice containing approximately 60 copies of the transgene per genome in the heterozygous state was produced. Unless otherwise indicated, homozygous transgenic mice were used in all studies. The integrity of the transgene in the genomic DNA was confirmed by Southern blot hybridization, after digestion with Not I. All breeding was done with C57Bl/6N mice (Taconic, NY, USA). The Tie2hABCA1 transgene was transferred after 5 generations onto Abca1 knockout (AbcA1-Ko) mice (Jackson Laboratory, Stock Number 003897) and ApoE-Ko mice (Jackson Laboratory, Stock Number 002052). Sibling controls were used in the analysis of the effect of the hABCA1 transgene in AbcA1-Ko and ApoE-Ko mice. In all other cases, C57Bl/6N mice were used as controls.

Gene expression analysis

For RNA isolation, tissue samples preserved in RNAlater (Invitrogen, Cat. No. AM7020) were homogenized in TRIzol Reagent (Invitrogen, Cat. No. 15596026), in a
Precellys24 (Bertin Technologies, France). RNA was isolated with the Invitrogen PureLink RNA Mini Kit (Cat. No. 12183018A), after DNase treatment. RNA concentration and integrity was evaluated with a NanoDrop and an Agilent 2100 analyzer, using RNA 6000 Pico Chip kits. RNA typically had an A260:A230 ratio greater than 1.7; an A260:A280 ratio of approximately 2.1 ± 0.1 and a RIN number of 8.2±0.2.

RNA (2 µg) was reverse transcribed, using ABI TaqMan Reverse Transcriptase Reagents Kit. Real-time qPCR assays were performed with an ABI 7900HT, with 40 ng of cDNA per reaction. A list of ABI TaqMan assays used in the study is shown in Supplementary Table 1. For expression analysis of mouse 28S rRNA, the following primers and TaqMan MGB probe were custom designed, using ABI Primer Express 3.0 software: forward primer: TCTGCCCAGTGCTCTGAATG; reverse primer: CGTTTACCAGCGCTTCAT; fluorogenic probe with FAM and quencher: CAAAGTGAAGAAATTC.

A calibration curve based on the expression of mouse Abca1 gene in the kidney was constructed and used to determine the relative expression of hABCA1, as well as other genes. Normalization of expression data was done by using results for expression of mouse β-actin and 28S rRNA genes. In the latter case, concentration of cDNA in PCR wells was decreased by 512-fold in comparison with test samples, in order to match the level of expression. The presence of contaminating DNA was excluded by performing no reverse transcription controls. SDS 2.2.1 software from ABI7900HT was used for calculations. The relative level of expression was also measured by comparative C_T (ΔΔC_T) method (24), with mouse β-actin, 18S or 28S rRNA genes as reference genes.

Gene expression was also determined with Endothelial Cell Biology PCR Array PAMM-015A from SA Biosciences. A complete list of the genes included in the Array can be found in Supplementary Tables 2.

Western blot analysis was done, as previously described (25), with an anti-ABCA1 antibody from Abcam (ab18180), which reacts with ABCA1 from both human and mouse tissue. Anti-human/mouse β-actin was used as a control.

Animal studies
Mice were housed under controlled conditions, with a 12/12 h light/dark cycle, and fed either a standard rodent autoclaved chow diet, containing 4.0% fat (NIH31 chow diet; Zeigler Brothers Inc., Gardners, Pennsylvania, USA), or the Paigen diet (a cocoa butter diet TD90221, Harlan Teklad, Madison, WI), containing 1.25% cholesterol and 0.5% cholic acid (HFHC diet). For evaluation of atherosclerosis, en face measurements of the surface of mouse aortas covered by lipid deposits were performed, as previously described (26). Quantification of aortic plaques was done in a blinded fashion, using the Image-Pro Plus version 4.1 software (Media Cybernetics, Inc., MD). All animal experiments were approved by the Animal Care and Use Committee of the NHLBI (#H-0050R2).

Analyses of plasma lipids, lipoproteins

Total cholesterol, triglycerides, phospholipids, and free cholesterol in EDTA-plasma were quantified after a 4-h fast, using enzymatic kits, as described elsewhere (27). HDL-C was determined after precipitation of apoB-containing lipoproteins, with Raichem HDL-Cholesterol reagent.

Cell culture studies

Resident mouse peritoneal macrophages were obtained after peritoneal lavage (28), with 10 ml of sterile PBS without Ca\(^{2+}\) and Mg\(^{2+}\), containing 50 µg/ml of heparin. Cells from 10 mice were placed into 6-well tissue culture plates and allowed to adhere for 1.5 h in serum-free DMEM or RPMI-1640, after which non-adherent cells were removed by rinsing with PBS. Adhered cells were directly used for RNA isolation. Mouse aortic EC were isolated from aorta tissue explants, following published protocols (29, 30). Mouse lung EC for efflux studies were isolated by a immunomagnetic method (31), using two rounds of selection (see Supplemental Material for detailed protocols). Cultured mouse lung and aortic EC, which were approximately 90% pure as determined by staining with Dil-acetylated LDL (32), had a characteristic cobblestone appearance when confluent, and formed tube-like structures when pre-confluent on dishes covered by Poly-D-lysine. Both aortic and lung EC were used at passage numbers less than 5. Normal human primary aortic EC (ATCC number PCS-100-011) and human
endothelium-derived EA.hy926 cells (ATCC number CRL-2922) were grown according to supplier’s recommendations.

In order to minimize possible effects of cultivation of EC on gene expression, EC were also isolated for gene expression studies after only one round of immunomagnetic selection, using the approach described above or by a rapid method shown below to yield highly purified EC. Minced mouse lungs were incubated with collagenase (2 mg/ml) in 25 ml of DMEM (Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, with gentle agitation every 5 to 8 min. Lung cells were then separated by 12 passes through a 14 gauge blunt-ended needle and then passed through a 70 micron disposable cell strainer into 15 ml of high glucose DMEM containing 20% fetal calf serum. Cells were pelleted by centrifugation at 400 g for 8 min at 4°C and re-suspended in 1 ml of cold PBS with 0.1% BSA. Cells were incubated with anti-ICAM-2 antibody-conjugated magnetic beads for 12 minutes at RT on a rotating platform. The anti-ICAM-2 beads were prepared by mixing a rat, anti-mouse, FITC-conjugated CD102 antibody (BD Pharmingen, Franklin Lakes, NJ) with sheep anti-rat IgG magnetic beads (Invitrogen, Carlsbad, CA). Vascular EC were then isolated with a Magnetic Particle Concentrator (DynaMag™-2, Invitrogen) and re-suspended in 1 ml of PBS with 0.1% BSA. A total of 5 rounds of cell purification were performed and EC were re-suspended in 500 µl of PBS with 0.1% BSA. Isolated vascular EC were shown by flow cytometry to have 95% purity and 85% viability, using the FITC-conjugated anti-CD102 antibody and 7-AAD.

Cholesterol efflux to apoA-I and HDL was measured in quadruplicate, in at least two independent experiments, as previously described (33). Cells were labeled by incubation for 24 h in endothelial Growth Media (see Supplemental Material), with 10% FCS and 1 µCi/ml of [1,2-3H]cholesterol (PerkinElmer). Cells were washed two times with Dulbecco’s PBS, without Ca2+ and Mg2+, and cholesterol efflux to apoA-I or HDL diluted in serum-free growth media, supplemented with 0.1% BSA, was monitored after 18 h.

Statistical analysis

Unless otherwise indicated, all data were analyzed by Student’s t-test with GraphPad PRISM version 5.03 software. Statistically significant differences were
defined as a two-tailed probability of less than 0.05. Values for efflux studies are expressed as mean ± SD. In all other cases, results are presented as means ± SEM.
RESULTS

Expression of hABCA1 transgene and endogenous genes

Except for the liver, hABCA1 expression was observed in all tissues tested. Using total tissue homogenates, the level of hABCA1 mRNA expression on a normal chow diet compared to the endogenous murine Abca1 gene ranged between 1-12%, depending on the tissue (Fig. 1A). Because EC typically comprise less than 1-2% of all cells in most tissues (1, 2), the ABCA1 expression values from total tissue homogenates is an underestimate of the level of hABCA1 expression in endothelial cells. In order to better examine this issue, we compared the level of hABCA1 in EC isolated from the lung of transgenic mice, human primary aortic EC and the human EA.hy926 endothelial cell line and found a 4.5-11-fold higher level of mRNA for hABCA1 in the cells from the transgenic mice (Fig. 1B). When we compared the level of hABCA1 to mAbcA1 in isolated lung EC from transgenic mice, we observed an approximate 2-fold increase in the enrichment of hABCA1 compared to what was observed for total lung tissue homogenates (Fig. 1A). The level hABCA1 mRNA, however, was only 10.3 ± 3% relative to that of mAbcA1 in isolated lung EC. By Western blot analysis of primary cultures of aortic EC, the total level of ABCA1 protein was 1.7-fold higher (P< 0.05) in transgenic mice versus control mice (Fig. 1C).

Tie2 promoter constructs, containing a longer promoter segment than used in this study, have also been reported to be active in macrophages of transgenic mice (34). In order to confirm the endothelial specificity of hABCA1 expression, we compared the level of hABCA1 in lung EC and peritoneal macrophages (Fig. 2A). The relative expression of hABCA1 in lung EC was more than 80-fold higher than in peritoneal macrophages, which only showed trace expression of hABCA1. These results are consistent with the known endothelial specificity of the 2.1 kb-Tie2 promoter coupled with the 1.6 kb-Tie2 enhancer used in this study (22, 23).

The expression of the hABCA1 transgene in EC of mice did not significantly alter the expression of endogenous mouse Abca1, Abcg1 or Sr-b1 genes in most tissues tested, or in isolated lung EC and peritoneal macrophages (Table 1, compare also Fig. 2B and 2C). Only in the kidney of transgenic animals was the expression of mouse
Abca1 significantly altered (1.8-fold increase; \( P=0.01 \), Table 1). It is interesting to note that the level of Abca1 expression in macrophages of normal mice was relatively high compared to lung EC (Fig. 2B) (7.7 ± 0.9 fold increase; \( P<0.002 \)). In contrast, Sr-b1 was more abundantly expressed in EC compared to macrophages (17.6 ± 3.4 fold increase; \( P<0.03 \)). Lung EC and macrophages, however, had a similar high level of Abcg1 expression in both normal and transgenic mice, which was 3 ± 0.7 fold higher than in liver (\( P<0.001 \)) (Fig. 2D). We also examined the level of apoA-I gene expression in isolated lung EC, peritoneal macrophages and aorta, because apoA-I has been reported to be produced by endothelial cells lining the blood brain barrier (35, 36), but only trace amounts of apoA-I mRNA were detected in these tissues and cells (data not shown).

**Effect of hABCA1 transgene on cholesterol efflux**

The functional impact of hABCA1 expression in EC was evaluated by cholesterol efflux studies (Fig. 3). Experiments with primary cultures of aortic EC isolated from Tie2hABCA1 transgenic and C57Bl/6N control mice showed that overexpression of hABCA1 transgene in EC was accompanied by a 2.6 ± 0.6 fold increase in cholesterol efflux to apoA-I (\( P<0.00001 \)) (Fig. 3A). No difference in the amount of cholesterol efflux between control and transgenic aortic EC was observed when HDL was used as an acceptor.

To examine just the role of the transgene in cholesterol efflux, EC were isolated from Tie2hABCA1 x AbcA1-Ko mice, as well as from AbcA1-Ko mice and wild-type C57Bl/6N control mice (Fig. 3B). Because we were unable to obtain aortic EC from tissue explants from AbcA1-Ko mice, only lung EC were used for this part of the study. As expected, cholesterol efflux to apoA-I was not observed from lung EC isolated from AbcA1-Ko mice. Cholesterol efflux, however, was approximately 30% higher (\( P<0.03 \)) for cells from Tie2hABCA1 x AbcA1-Ko versus control mice. No significant differences were observed when HDL was used as an acceptor, except for a modest decrease in cholesterol efflux from cells isolated from AbcA1-Ko mice.

**Effect of hABCA1 transgene on plasma lipids**
Tie2hABCA1 transgenic mice had approximately a 25% increase \( (P<0.0001) \) in plasma HDL-C compared to control mice on a normal chow diet (Table 2). They also had slightly higher plasma phospholipids, total cholesterol, and lower triglycerides. On apoE-Ko or AbcA1-Ko background, the hABCA1 transgene did not, however, significantly alter plasma HDL-C (Table 2). In transgenic mice on AbcA1-Ko background, the level of plasma triglycerides and phospholipids was also increased in comparison with non-transgenic sibling control but no effect from the transgene was observed on HDL-C, which was undetectable in both the transgenic Tie2hABCA1 x AbcA1-Ko mice and the AbcA1-Ko mice (Table 2). When placed on a HFHC diet for 6 months, Tie2hABCA1 transgenic mice had 40% higher \( (P<0.003) \) HDL-C than control mice (Table 2), but no other significant lipid changes were observed. In the absence of endogenous AbcA1, lipid profiles of plasma taken from the transgenic mice after 1.5 month on HFHC diet did not significantly differ from non-transgenic sibling control (data not shown).

**Effect of hABCA1 transgene on atherosclerosis**

After 6 months on a HFHC diet, Tie2hABCA1 transgenic mice had approximately 40% less \( (P<0.02) \) aortic surface lesions compared to C57Bl/6N control mice (Fig. 4A). The effect of the hABCA1 transgene on atherosclerosis was also confirmed in a second study, when Tie2hABCA1 transgenic mice and their non-transgenic sibling controls were placed on HFHC diet for 3 months. Because of relatively short time of this study, only a limited amount of atherosclerosis, however, was observed (Supplemental Fig. 1). When the Tie2hABCA1 transgene was transferred onto an AbcA1-Ko background, however, no significant atheroprotection from the transgene or change in plasma lipids was observed (Fig. 4B). Similarly, no effect of the hABCA1 transgene was observed in plasma lipids (Table 2) or in the level of atherosclerosis when expressed in mice on an apoE-Ko background (Fig. 4C).

**Effect of HFHC diet on expression of hABCA1 and endogenous genes**

The HFHC diet for 3-months significantly induced in control mice the expression of mouse \( Abca1 \) and \( Abcg1 \) in several different tissues, but particularly in the liver and
kidney (Fig. 5A). In contrast, endogenous Sr-b1 did not show major gene expression changes in response to the diet. Overall, the gene expression changes observed from the HFHC diet treatment in the Tie2hABCA1 transgenic mice were more blunted compared to control mice, particularly for mAbcA1 (Fig. 5B). Only changes for increased expression of Abcg1 in heart and liver and Sr-b1 in the liver were statistically significant. As expected, the HFHC diet also did not cause major changes in the tissue expression of the hABCA1 transgene when under the control of the heterologous Tie2 promoter (Fig. 1A).

Aortic gene expression was also analyzed after 3-months on a HFHC diet, using RT² Profiler PCR array system for genes related to endothelial cell function (see Supplementary Tables 2 and 3). The expression of 9 out of the 84 examined genes was found to be changed in Tie2hABCA1 transgenic mice compared to control mice (Fig. 6A). Two of the three genes, with increased expression in transgenic mice, namely the Ras homolog gene family, member B (Rhob), which is involved in angiogenesis and EC survival (37), and Tissue factor pathway inhibitor (Tfpi), an anti-coagulant factor protein (38) are known to be anti-atherogenic. No difference was observed after HFHC diet for the expression of the atheroprotective eNOS gene (39), but it was 2.2-fold more expressed in Tie2hABCA1 transgenic mice compared to control mice on a normal chow diet (Fig. 6B). Several of the remaining 6 genes with decreased expression in the aortas of transgenic mice, namely Casp6, Cxc11, Pecam1, Ripk1, Sod1 and Tnfsf10 (Fig. 6A), are known to be down-regulated in normal aortas compared to atherosclerotic plaques (40-42).

**DISCUSSION**

The two main findings of this study are that increased endothelial specific expression of hABCA1 was associated with increased HDL-C and protected against diet-induced atherosclerosis in C57Bl/6N mice. These changes were observed despite the fact that the level of expression of hABCA1 in aortic EC of transgenic mice was relatively modest (Fig. 1). Based on Western blot analysis, there was a 1.7-fold increase in total ABCA1 protein in aortic EC from transgenic mice compared to control mice (Fig. 1C), which is similar to the induction, observed for endogenous mouse AbcA1 mRNA in the aorta of
mice placed on a HFHC diet (Fig. 5). Level of hABCA1 mRNA in lung EC from transgenic mice was significantly higher (4.5-11-fold increase) compared to cultured human primary aortic cells or the EA.hy926 human endothelial cell line (Fig. 1B). In contrast, hABCA1 mRNA was present at approximately 10% of the level of mouse AbcA1 in lung EC isolated from transgenic mice. Some of these apparent differences in the level of hABCA1 are likely due to rapid gene expression changes in cells once isolated from tissues and or perhaps differences in the expression of genes in endothelial cells from different tissues (2, 43). Besides having a different promoter, the transgene also had a heterologous SV40 3' UTR, which could have potentially improved the translation of the transgene transcript compared to the endogenous gene. Based on the cholesterol efflux results, the level of increased expression of hABCA1 in the aortic EC from transgenic mice was enough to increase cholesterol efflux to apoA-I by approximately 2.6-fold (Fig. 3A) and to restore cholesterol efflux to normal levels in lung EC when on the AbcA1-Ko background (Fig. 3B). Overall, these results are consistent with only a relatively low level of hABCA1 transgene expression in EC but within a physiologic range and sufficient to alter cholesterol efflux.

Besides AbcA1, mouse EC were also found to express AbcG1 and Sr-b1 (Fig. 2B and C), which can also contribute to cholesterol efflux. From data presented in Fig. 2D and Table 1, it is evident that lung EC had relatively high level of expression of AbcG1, which was close to the level found in peritoneal macrophages and exceeded that found in the liver by approximately 3-fold. Lung EC had especially high level of Sr-b1 expression, which was 18-fold higher than in macrophages and was comparable to the level observed in the liver (Fig. 2B, C and D). This observed pattern of relatively high expression of these other transporters, probably reflects their role in cholesterol trafficking of EC. As can be observed in Fig. 5, the expression of the hABCA1 transgene appeared to blunt the induction of the endogenous Abca1 gene in the aorta of the transgenic mice when placed on a HFHC diet but did not affect the expression of the other endogenous genes that can modulate cholesterol efflux, namely Abcg1 and Sr-b1.

Tie2hABCA1 transgenic mice were observed to have a 25% increase in HDL-C when on a normal chow diet compared to control mice (Table 2). Interestingly, the transgene in EC was only able to influence the level of HDL-C in the presence of the
endogenous mouse AbcA1 gene or, in other words, only when the liver and intestine were able to produce nascent HDL, which then interacts with ABCA1 in peripheral cells (14, 15, 44). When the Tie2hABCA1 transgene was transferred onto AbcA1-Ko mice, HDL-C was still very low (Table 2), despite the fact that \textit{in vitro} isolated EC from these mice were able to efflux cholesterol to apoA-I (Fig. 3B). Unlike brain capillary EC, which are able to express and secrete apoA-I (35, 36), the expression of apoA-I in EC from other sites was undetectable, which could account for the dependence of EC for nascent HDL produced by the liver and intestine to act as an acceptor for cholesterol efflux stimulated by ABCA1 in EC. Endothelial expression of hABCA1 transgene was also unable to affect the level of plasma HDL-C in apoE-deficient mice (Table 2). It was previously shown that the expression of the hABCA1 transgene in liver of apoE-Ko mice also has a minimal effect on plasma lipids, and in fact, increased aortic lesion area compared to apoE-Ko animals (45). The mechanism for the low levels of HDL-C in apoE-Ko mice is not known, but apoE has been proposed to play an important role in the biogenesis and metabolism of HDL, at least in mice (46, 47).

In this study the effect of Tie2hABCA1 transgene was specifically determined in lung and aortic EC, where the transgene increased level of expression of ABCA1 and cholesterol efflux to apoA1 (fig. 1C and Fig.3). EC from different areas of the vasculature can display remarkable heterogeneity in structure and function (2, 43). The Tie2 promoter is known, however, to be expressed in most EC (22, 23), but whether increased ABCA1 expression in a particular tissue or site in the vasculature was more critical to the observed changes in HDL metabolism or atheroprotection was not assessed in this study.

Tie2hABCA1 transgenic mice were also observed to have decreased atherosclerosis when placed on HFHC diet compared to control mice (Fig. 4A). This may be related to the 40% increase in HDL-C in the transgenic mice on the HFHC diet (Table 2). When the Tie2hABCA1 transgene was transferred to the AbcA1-Ko or apoE-Ko mice and no changes were found in HDL-C, there was no apparent protection from the transgene on atherosclerosis (Fig. 4B and C). Besides the effect of HDL in promoting cholesterol efflux, the increased HDL in the transgenic mice could also have reduced atherosclerosis by some of the other beneficial properties of HDL (48). For
example, HDL has been shown to reduce inflammation by suppressing activation of NF-kappa B, and also by decreasing the expression of adhesion proteins (48-50). We observed that in the aortas of the Tie2hABCA1 transgenic mice on a normal chow diet, but not the HFHC diet, there was increased expression of eNOS (Fig. 6B), which is known to have several anti-atherogenic effects (49, 51). Caspase 6, Ripk1 (receptor (TNFRSF)-interacting serine-threonine kinase 1) and Tnfsf10 (Tumor necrosis factor (ligand) superfamily, member 10) were found to be down-regulated in the Tie2hABCA1 transgenic mice (Fig. 6A) and are known to play important roles in programmed cell death or apoptosis (40-42). The expression of Cxcl1 (chemokine (C-X-C motif) ligand 1) and Pecam1 (platelet/EC adhesion molecule 1), which promote transendothelial migration of leukocytes and vascular inflammation (52, 53) were also down-regulated. In contrast, the expression of the anti-atherogenic genes Rhob, which is involved in angiogenesis and EC survival (37) and Tfpi, an anti-coagulant factor protein (38) were increased in Tie2hABCA1 transgenic mice compared to control mice on a HFHC diet (Fig. 6A).

It is known that a number of inflammatory disorders, such as systemic vasculitis, Kawasaki disease and Behcet’s disease, are associated with low HDL, endothelial dysfunction and pro-atherogenic gene expression (54-57). Endothelial dysfunction can also occur in diabetes (58) and hypertension (59), and is often also associated with decreased HDL-C (60, 61). The mechanistic link between endothelial dysfunction and HDL-C is not clear but is likely related to increased production of inflammatory cytokines and their effect in suppressing HDL production and accelerating its catabolism (62). Low HDL can then further negatively impact on endothelial function, leading to the rapid progression of atherosclerosis (63, 64). Results from this study, therefore, suggest that endothelial expression of ABCA1 can perhaps play an important role in these diseases and in this complex interrelationship between endothelial dysfunction, low HDL and atherosclerosis.

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pSPTg.T2FpAXK plasmid, Dr. Yoshitaka Sekine for help with Western blot experiments, and Dr. Chengyu Liu for help in creating the transgenic mice.
**TABLE 1.** Relative expression of mouse AbcA1, AbcG1 and Sr-b1 genes in tissues and peritoneal macrophages in transgenic and control female mice.

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>Gene</th>
<th>Relative expression (in %)</th>
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<tr>
<td></td>
<td></td>
<td>Aorta</td>
<td>Heart</td>
<td>Kidney</td>
<td>Liver</td>
<td>Lung</td>
<td>Spleen</td>
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<tr>
<td>Normal (N=3)</td>
<td>AbcA1</td>
<td>393 ± 71</td>
<td>192 ± 43</td>
<td>100 ± 13</td>
<td>298 ± 88</td>
<td>256 ± 69</td>
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<td>Transgenic (N=3)</td>
<td></td>
<td>472 ± 22</td>
<td>202 ± 29</td>
<td>178 ± 13</td>
<td>376 ± 24</td>
<td>224 ± 16</td>
<td>173 ± 33</td>
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<tr>
<td>Difference (p)</td>
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<tr>
<td>Normal (N=3)</td>
<td>AbcG1</td>
<td>145 ± 73</td>
<td>60 ± 31</td>
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<td>60 ± 3</td>
<td>435 ± 120</td>
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<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Normal (N=3)</td>
<td>Sr-b1</td>
<td>358 ± 17</td>
<td>186 ± 2</td>
<td>98 ± 12</td>
<td>552 ± 37</td>
<td>396 ± 81</td>
<td>163 ± 21</td>
</tr>
<tr>
<td>Transgenic (N=3)</td>
<td></td>
<td>367 ± 58</td>
<td>236 ± 18</td>
<td>118 ± 8</td>
<td>451 ± 23</td>
<td>272 ± 18</td>
<td>193 ± 43</td>
</tr>
<tr>
<td>Difference (p)</td>
<td></td>
<td>NS</td>
<td>0.05</td>
<td>NS</td>
<td>NS</td>
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</table>

Tissues from normal C57Bl/6N females (N=3) or transgenic females (N=3) or peritoneal macrophages from C57Bl/6N males (N=10) and transgenic males (N=11) were analyzed in duplicate in two independent experiments. All results are normalized to expression of mouse AbcA1 in normal kidneys, which was assigned a value of 100%. In two independent experiments (N=6 in each case), no significant difference was found in the expression of the hABCA1 transgene, AbcG1 and Sr-B1 between females and males for peritoneal macrophages.
### TABLE 2. Plasma lipid profiles in transgenic and control females on normal chow and HFHC diets.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TC</th>
<th>TG</th>
<th>PL</th>
<th>FC</th>
<th>CE</th>
<th>HDL-C</th>
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<tbody>
<tr>
<td>Normal chow diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tie2hABCA1 C57Bl/6N, N=17-49</td>
<td>81.4 ± 2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.8 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>179.5 ± 4.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27.1 ± 3.6</td>
<td>67.5 ± 3.8</td>
<td>66.3 ± 1.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C57Bl/6N, N=26-38</td>
<td>74.6 ± 2.0</td>
<td>85.3 ± 3.3</td>
<td>157.1 ± 3.2</td>
<td>20.3 ± 0.8</td>
<td>62.3 ± 2.4</td>
<td>53.0 ± 1.5</td>
</tr>
<tr>
<td>After 6 month on HFHC diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tie2hABCA1 C57Bl/6N, N=13</td>
<td>302.8 ± 16.4</td>
<td>32.9 ± 1.7</td>
<td>312.2 ± 25.4</td>
<td>164.2 ± 17.7</td>
<td>138.6 ± 8.7</td>
<td>56.0 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C57Bl/6N control, N=9</td>
<td>302.3 ± 23.9</td>
<td>31.7 ± 2.7</td>
<td>264.2 ± 16.9</td>
<td>138.9 ± 7.8</td>
<td>163.4 ± 21.2</td>
<td>40.5 ± 3.5</td>
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<tr>
<td>Normal chow diet</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tie2hABCA1 x AbcA1-Ko, N=11-15</td>
<td>10.1 ± 1.1</td>
<td>106.6 ± 8.7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>54.3 ± 5.0&lt;sup&gt;f&lt;/sup&gt;</td>
<td>6.6 ± 0.8</td>
<td>3.6 ± 0.7</td>
<td>&lt;3</td>
</tr>
<tr>
<td>Sibling control AbcA1-Ko, N=6-9</td>
<td>8.1 ± 1.3</td>
<td>81.0 ± 2.5</td>
<td>36.8 ± 2.5</td>
<td>4.3 ± 0.9</td>
<td>2.9 ± 1.2</td>
<td>&lt;3</td>
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<tr>
<td>Normal chow diet</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tie2hABCA1 x ApoE-Ko, N=20</td>
<td>551.6 ± 31.3</td>
<td>90.0 ± 5.2</td>
<td>341.3 ± 21.1</td>
<td>264.6 ± 18.2</td>
<td>287.1 ± 21.4</td>
<td>9.0 ± 0.8</td>
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<td>Sibling control ApoE-Ko, N=25-32</td>
<td>537.4 ± 25.7</td>
<td>92.8 ± 3.9</td>
<td>343.6 ± 14.5</td>
<td>273.2 ± 11.0</td>
<td>264.2 ± 20.4</td>
<td>7.6 ± 0.7</td>
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</table>

All values are expressed in units of mg/dL.<sup>a</sup>P<0.04; <sup>b</sup>P<0.003; <sup>c</sup>P<0.0002; <sup>d</sup>P<0.0000001; <sup>e</sup>P<0.015; <sup>f</sup>P<0.005. Used abbreviations: TC – total cholesterol; TG – triglycerides; PL – phospholipids; FC – free cholesterol; CE – cholesteryl ester.
FIGURE LEGENDS

Figure 1. Analysis of expression of Tie2hABCA1 in transgenic mice.  
(Panel A) Expression of hABCA1 in tissues of Tie2hABCA1 transgenic mice relative to the expression of endogenous Abca1 in the same tissues (in %). RNA from the indicated organs were isolated from transgenic females kept on normal chow diet (N=5, open bars) or on a 3-month HFHC diet (N=3, solid bars). Differences between expression hABCA1 on normal chow and HFHC diet were not statistically significant, \( P>0.3 \). (Panel B) Expression of hABCA1 mRNA in lung EC isolated from Tie2hABCA1 transgenic mice related to expression of ABCA1 in human primary aortic EC (hPAEC) or human endothelium-derived EA.hy926 cells. (N=3 in each case); \( *P<0.0003 \). (Panel C) Western blot analysis of protein extracts from primary cultures of aortic EC (AEC) isolated from Tie2hABCA1 transgenic mice versus control C57Bl/6N mice. Results are the mean of triplicates and were normalized to \( \beta \)-actin. \( *P<0.05 \).

Figure 2. Relative gene expression of ABCA1 and endogenous genes. 
mRNA levels for hABCA1, AbcA1, AbcG1 and Sr-b1 were determined from the mean of two independent experiments from isolated lung EC, liver and resident peritoneal macrophages (PM). Comparative Ct method was used for calculation of gene expression. Each time lowest level of expression was presented as 1 unit. (Panel A) Expression of hABCA1 in lung EC relative to the expression in PM, also isolated from the transgenic mice. \( *P<0.00001 \). (Panel B) Relative expression of the 3 genes in lung EC and PM isolated from normal C57Bl/6N mice. \( *P<0.003 \). (Panel C) The same as in panel B but from cells isolated from the transgenic mice. \( *P<0.0005 \). (Panel D) Relative expression of the 3 genes in lung EC and liver isolated from normal C57Bl/6N mice. \( *P<0.03 \).

Figure 3. Effect of ABCA1 on cholesterol efflux.  
(Panel A) Cholesterol efflux was measured from aortic EC isolated from Tie2hABCA1 transgenic mice and control C57Bl/6 mice. Efflux was monitored 18 h after the addition of either apoA-I (10 µg/mL) or HDL (50 µg/mL), as described in Material and Methods part. \( *P<0.0003 \). (Panel B) Cholesterol efflux was measured as described above from
lung EC isolated from either control C57Bl/6N mice, Tie2hABCA1 x AbcA1-Ko or AbcA1-Ko mice.* $P=0.05$; ** $P=0.03$; *** $P=0.001$; **** $P<0.00001$.

**Figure 4. Effect of EC specific overexpression of ABCA1 on atherosclerosis.**
Percent of aortic lesions, as determined by *en face* analysis, is shown (Panel A) for Tie2hABCA1 transgenic mice (N=13) versus C57Bl/6N controls (N=11) on HFHC diet for 6 months (*$P<0.02$), (Panel B) for Tie2hABCA1 x AbcA1-Ko mice (N=7) versus non-transgenic sibling controls (N=4) on HFHC diet for 1.5 months ($P>0.7$) and (Panel C) for 8-month old Tie2hABCA1 x ApoE-Ko mice on normal chow diet (N=16) versus non-transgenic sibling controls (N=21) ($P>0.4$).

**Figure 5. Effect of HFHC diet on relative gene expression of endogenous genes.**
Control C57Bl/6N mice (N=3) (Panel A) and Tie2hABCA1 transgenic mice (N=3) (Panel B) were placed either on a normal chow diet or on a HFHC diet for 3 months and the mRNA levels for AbcA1 (open bars), AbcG1 (solid bars) and Sr-b1 (horizontal lined bars) were determined. Results are expressed as the ratio of the level of expression on HFHC diet over the normal chow diet for the indicated organs. *$P<0.05$; ** $P<0.02$.

**Figure 6. Effect of ABCA1 transgene on aortic gene expression.**
(Panel A) RNA from aortas of Tie2hABCA1 transgenic (N=3) and control C57Bl/6N (N=3) females on a HFHC diet for 3 months were independently analyzed by PAMM-015A (SA Biosciences) array with 84 genes. Results are expressed as the ratio of gene expression for Tie2hABCA1 transgenic mice over control mice (up-regulation). In cases when this ratio was $<1$, it was presented as negative ratio transgenic/control (down-regulation). *$P<0.05$; ** $P\leq 0.02$. (Panel B) RNA from aortas of Tie2hABCA1 transgenic (N=3) and control C57Bl/6N (N=3) females on a normal chow diet or after 3 months on HFHC diet were analyzed for eNOS gene expression. Comparative Ct method was used in calculations. *$P<0.04$. 


Figure 1

A) Expression of hABCA1 in % to mABCA1

- Normal chow diet
- HFHC diet

B) Fold difference in expression of hABCA1

- Lung EC/hPAEC
- Lung EC/EA.hy926

* Significant difference
Figure 1

C

![Image showing Western blot results for ABCA1 and β-actin](image)

**Relative ABCA1 protein concentration (%)**

- Control AEC
- hABCA1 AEC

*Statistical significance indicated by *
Figure 2

A

Expression Ratio of hABCA1 in Lung EC to PM

Lung EC
PM

B

Fold difference

Abca1 Abcg1 Sr-b1

Lung EC, Norm PM, Norm

*
Figure 2

C

Fold difference

Abca1 Abcg1 Sr-b1

Lung EC, Transgenic
PM, Transgenic

D

Fold difference

Abca1 Abcg1 Sr-b1

Lung EC, Norm
Liver, Norm
Figure 3

A

Cholesterol efflux (%)

apo-A1 HDL

Control line Tie2hABCA1

B

Cholesterol efflux (%)

apo-A1 HDL

C57Bl/6N Tie2-hABC1 x AbcA1-Ko AbcA1-Ko

* ** *** ****
Figure 4

A. Aortic area with lesions in %

B. Aortic lesions, %

Control Abca1-Ko Tie2hABCA1 x Abca1-Ko
Figure 4

Control apoE-Ko

Tie2hABCA1 x apoE-Ko

Aortic lesions, in %
Figure 5

A

Fold difference (HFHC diet/Normal chow diet)

mABCA1  mABCG1  SR-B1

Aorta  Heart  Kidney  Liver  Lung  Spleen

B

Fold difference (HFHC diet/Normal chow diet)

mABCA1  mABCG1  SR-B1

Aorta  Heart  Kidney  Liver  Lung  Spleen
Figure 6

A

Fold difference Tie2hABCA1/Control

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal diet</th>
<th>HFHC diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ace</td>
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</tr>
<tr>
<td>Casp6</td>
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B

Ratio Transgenic/Control

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<tr>
<th>Diet</th>
<th>Ratio</th>
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<tr>
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<td>HFHC diet</td>
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REFERENCES


